UNIVERSIDAD AUTÓNOMA DE MADRID

FACULTAD DE MEDICINA

DEPARTAMENTO DE MEDICINA

DETERMINANTES ESTRUCTURALES DE LA ACTIVIDAD BIOLÓGICA DE FAS LIGANDO SOLUBLE EN LA LESIÓN PULMONAR AGUDA

STRUCTURAL DETERMINANTS OF THE BIOLOGICAL ACTIVITY OF SOLUBLE FAS LIGAND IN ACUTE LUNG INJURY

MEMORIA PRESENTADA PARA OPTAR AL GRADO DE DOCTOR POR

Raquel Herrero Hernández

Bajo la dirección de los Doctores:

Thomas R. Martin

Gustavo Matute-Bello

Manuel Jiménez Lendinez

Madrid 2009

A mi familia, en especial a mi madre
para quien mi introducción a la investigación
ha significado mi ausencia durante estos últimos años.

AGRADECIMIENTOS

Quiero expresar mi admiración y más sincero agradecimiento a mis directores de Tesis: al Dr. Thomas R. Martin por acogerme en su laboratorio, hacerme crecer intelectualmente y darme siempre su apoyo incondicional; al Dr. Gustavo Matute-Bello por ser una persona clave en mi carrera y en mi proceso de aprendizaje; al Dr. Manuel Jiménez Lendínez por su atención y apoyo durante mis años de residencia en Medicina Intensiva.

Este trabajo ha sido posible por la participación de muchas personas, a las que también quisiera dar las gracias:

- Al Dr. Thomas R. Martin, de quien admiro tantas cosas en el aspecto humano como científico que no podría explicarlas con tan sólo unas pocas palabras. Muchas gracias por saber sacar siempre lo mejor de mí, iluminarme intelectualmente y hacerme sentir siempre apoyada.
- Al Dr. Gustavo Matute-Bello por transmitirme siempre su entusiasmo por el conocimiento científico y por servirme de ejemplo para seguir aprendiendo en el mundo de la investigación. Muchas gracias por ofrecerme siempre tu ayuda y apoyo.
- Al Dr. Manuel Jiménez Lendínez por haberme guiado y enseñado durante mis años de residencia en medicina intensiva, y por haberme transmitido importantes valores y pautas en la toma de decisiones, al pie de cama del enfermo crítico. Muchas gracias por tus clases de fisiopatología que nos ofrecías siempre a los residentes con gran entusiasmo.
- Al Dr. Roger F. Butterworth y al Dr. Alan S. Hazell por haber sido los primeros en abrirme las puertas a la investigación experimental, sin lo cual no habría ocurrido nada de lo que he aprendido y vivido en los últimos 4 años.
- Al Dr. Osamu Kajikawa por su amistad y amable paciencia para enseñarme las técnicas que han sido fundamentales en el desarrollo de este trabajo. Sin su contribución, este estudio no habría sido posible.
- A la Dra. Xiaoyun Fu por sus valiosas contribuciones en los análisis por espectrometría de masa y por compartir con nosotros sus amplios conocimientos en biología molecular. A John T. Ruzinsky por su amable contribución en la medición de citocinas por Luminex y a Alex W. Farnand por la realización de immunohistoquímica en el tejido pulmonar.
- A mis compañeros de laboratorio, en especial a Venus A. Wong por su incalculable ayuda en el desarrollo en los modelos animales, y por ser una gran amiga. A Mark

M. Wurfer, Anne B. Lipke, Kurayasu Kurahashi, Steve Mongovin, Lincoln Smith, Dong Chang, Reynout A. Bem por su ayuda en este trabajo, por sus consejos y por su apoyo a lo largo de este tiempo. Espero tener la suerte de seguir compartiendo con vosotros vuestra amistad y muchas más horas de laboratorio, de discusión de ideas y de experiencias.

- A las coordinadoras de investigación, Deborah Snapp, Diane Tomita y Pam Henderson por su ayuda. Gracias por ser siempre tan amables y tener la voluntad de ayudar a todo el personal del laboratorio. Sin duda hacéis que el trabajo sea mucho más fácil.
- A los pacientes, a mis compañeros de residencia y a mis adjuntos, de quienes tanto he aprendido. En especial a Eva Herrero y Paula Hidalgo por su amistad y por su apoyo en mi decisión de introducirme en el mundo de la investigación.
- A Javier, por compartir conmigo amor, trabajo, sacrificios, tantas ilusiones y años juntos. Muchas gracias por estar siempre a mi lado y hacer que todo sea más fácil. Espero tenerte siempre conmigo.
- A mi padre, quien supo despertar en mí el interés por la medicina, y siempre valoró el esfuerzo que supone dedicarse al estudio y a la práctica de esta disciplina.
- A mi madre, hermanos y sobrinos por ofrecerme su amor, comprensión y consejos en mis decisiones en la vida. A pesar de la distancia, siempre estais a mi lado.
- A Francisco Vaquero por darnos siempre a Javier y a mí palabras de ánimo, su apoyo y su ayuda a lo largo de todos estos años.
- A todos mis amigos, por su apoyo permanente a pesar de la distancia.

Este trabajo ha sido financiado por el National Institutes of Health (NIH HL-081764, HL-083044) y el VA Medical Research Service.

ABBREVIATIONS

aa, amino acid/s
Ab, antibody
ABGs, arterial blood gases
AFC, alveolar fluid clearance
ALI, Acute Lung Injury
Apaf-1, Apoptosis protease-activating factor-1
APC, activated protein C
ARDS, Acute Respiratory Distress Syndrome
ATP, adenosine triphosphate
AT1, angiotensin receptor subtype-1

BAL, Bronchoalveolar lavage
BALF, Bronchoalveolar lavage fluid
BSA, bovine serum albumin
b.w., body weight

Ca, calcium
CaM, Ca2+/calmodulin
CD95, Fas
CD95L, Fas Ligand
CFTR, Cystic fibrosis transmembrane conductance regulator
CH11, Fas-activating antibody
Cl, Chlorum
Cl^-, Chloride ion
cmH_2O, Centimeters of water
COPD, chronic obstructive pulmonary disease
CT, computed tomographic

DD, death domain
DED, death effector domain
DIC, disseminated intravascular coagulation
DNA, Deoxyribonucleic acid
DTT, dithiothreitol
DTPA, diethylene triamine pentaacetic acid

ECM, extracellular matrix
EDTA, ethylenediaminetetraacetic acid
ELISA, Enzyme-Linked ImmunoSorbent Assay
ENaC, amiloride-sensitive epithelial sodium channel
ENA, epithelial neutrophil activating peptide

FADD, Fas-Associated protein with Death Domain
FasL, Fas Ligand
FIO_2, Fraction of inspired oxygen
FLICE, FADD-like IL-1β-converting enzyme
FLIP, FLICE-like inhibitor protein
FN, fibronectin
FRC, Functional residual capacity

g, gram/s
GM-CSF, granulocyte–macrophage colony-stimulating factor
GSH, glutathione

h, hour
HCl, Hydrochloric acid
HEK, Human Embryonic Kidney
HIF-1, hypoxia inducible factor
HOCl, hypochlorous acid
H_2O_2, hydrogen peroxide
HOCl, hypochorous acid
HPLC, High Performance Liquid Chromatography
HRE, hypoxia response element
HRP, Horseradish peroxidase

IAM, iodoacetamide
IAP, inhibitory proteins of apoptosis
ICAM-1, inter-cellular Adhesion Molecule-1
ICE, activation of the IL-1β converting enzyme or Caspase-1
ICU, Intensive Care Unit
IFNG, interferon-δ
Ig, immunoglobulin
IGF1, insulin-like growth factor-1
IκB, inhibitor factor of NF-κB
IL, interleukin
IL-1RA, Interleukin-1 receptor antagonist
IPF, idiopathic pulmonary fibrosis

JKT, Jurkat
Jo-2, Fas-activating monoclonal antibody

K, potassium
KC, chemokine CXCL1
KDa, Kilodalton
kg, kilogram/s
KGF, Keratinocyte grow factor

LBP, lipopolysaccharide-binding protein
LDL, low-density lipoprotein
LPS, lipopolysaccharide

M, molar
μM, micromolar
μL, microliter
MCP, monocyte chemotactic peptides
Met, methionine
MetO, methionine sulfoxide
2-ME, 2-mercaptoethanol
Me_2S, Dimethyl sulfide
mFasL, membrane Fas Ligand

mg, milligram/s
min, minute/s
MIF, macrophage migration inhibitory factor
MIP, macrophage inflammatory protein
mL, milliliter/s
MLC, myosin light chain
MLCK, myosin light chain kinase
MLE-12, Murine Lung Epithelial-12
mmHg, millimeter of mercury
mM, milimolar
MMP, metalloproteinases
MOF, multiple organ failure
MODS, multiple organ dysfunction syndrome
MPO, myeloperoxidase
Msr, methionine sulfoxide reductase
MW, molecular weight

Na, Sodium
NaCl, sodium chloride
NADPH oxidase, nicotinamide adenine dinucleotide phosphate-oxidase
NF-κB, nuclear factor-κB
ng, nanogram/s
nM, nanomolar
NO, nitric oxide
NOS, nitric oxide synthases

O2-, superoxide anion radical
OH -, hydroxyl radical
ONOO -, peroxynitrate

PA, pulmonary artery
PAO_2, Partial alveolar pressure of oxygen
PAOP, pulmonary artery occlusion pressure
PaO_2, Partial arterial pressure of oxygen
$PaCO_2$, Partial arterial pressure of carbon dioxide
PAI, plasminogen activator inhibitor
PAGE, polyacrylamide gel electrophoresis
PBS, phosphate buffer saline
PCR, polymerase chain reaction
PEEP, positive end-expiratory pressure
pg, picogram/s
PMA, phorbol myristate acetate
PMN, Polymorphonuclear leukocytes

RNA, ribonucleic acid
RNS, reactive nitrogen species
ROS, reactive oxygen species
rh-sFasL, recombinant human soluble Fas Ligand

SA, self assembly

SDS, Sodium Dodecyl Sulfate Polyacrylamide
sFasL, soluble Fas Ligand
SIRS, systemic inflammatory response syndrome
SOD, superoxide dismutase
SP-A, Surfactant protein A

TFPI, tissue factor pathway inhibitor
TGF-α, transforming growth factor-alpha
TIMPs, tissue inhibitors of metalloproteinases
TNF, tumor necrosis factor
TNF(H), TNF homology
TNFR, TNF receptors
TRALI, Transfusion related acute lung injury
TUNEL, terminal dUTP nick-end labeling to detect apoptotic cells

VEGF, vascular endothelial growth factor
VILI, Ventilation-induced Lung Injury

WT, wild-type

INDEX

DETAILED INDEX

Page

Page

Page

LIST OF TABLES

Page

LIST OF FIGURES

Page

3. – RESUMEN EN ESPAÑOL

INTRODUCCIÓN

La Lesión Pulmonar Aguda (LPA) y su forma más grave, el Síndrome de Distrés Respiratorio Agudo (ARDS), es un síndrome frecuente y una causa importante de morbi-mortalidad en enfermos críticos. Se caracteriza por un daño alveolar difuso con aumento de la permeabilidad de la membrana alveolo-capilar y acumulación de edema alveolar proteináceo. El desarrollo del daño del epitelio alveolar es la principal característica de la LPA que determina la gravedad de la enfermedad y el pronóstico de estos pacientes.

El sistema formado por el receptor Fas y su ligando natural, Fas Ligando (FasL), parece estar implicado en la patogénesis de la LPA/SDRA. Fas ligando es una proteína que existe tanto unida a la membrana celular como en forma soluble. El FasL se une y agrega el receptor Fas en la superficie de la membrana celular de las células diana, e induce apoptosis y activación de repuestas inflamatorias. La forma soluble de FasL (sFasL) se genera principalmente a partir de la segmentación del FasL de membrana (mFasL) por la acción proteolítica de metaloproteinasas (MMPs) y otras enzimas.

En humanos, el sFasL está incrementado en el fluido de lavado bronquio-alveolar (LBA) en pacientes de riesgo para SDRA, así como en pacientes con SDRA establecido. Sin embargo, sólo el sFasL del fluido del LBA de pacientes con SDRA induce apoptosis en células epiteliales pulmonares humanas *in vitro*. Esta observación sugiere que existen factores que modulan la actividad de sFasL en el espacio aéreo de los pulmones de pacientes con LPA/SDRA.

Los mecanismos responsables de la bioactividad del sFasL no son bien conocidos. Se han identificado diferentes sitios de segmentación para la acción proteolítica de las metaloproteinasas -3 y -7 (MMP-3, -7) en la porción extracelular del mFasL, lo cual puede dar lugar a variantes estructurales del sFasL. Las dos formas principales de sFasL generadas de esta manera serían: una forma larga compuesta por el dominio TNF en el C-terminal y la región “stalk” en el N-terminal, y una forma corta formada solamente por el domino TNF. El dominio TNF media la unión del FasL al receptor Fas, pero la función de la región stalk se desconoce. El estado de agregación y de oxidación del sFasL son posibles factores que pueden modular la bioactividad del sFasL. Varias líneas de investigación sugieren que la bioactividad del sFasL depende

de su grado de agregación. Asimismo, es bien conocido que la oxidación puede alterar el estado de agregación y la actividad de múltiples proteínas. En los pulmones de los pacientes con LPA/SDRA existe un incremento de oxidantes por lo que muchas proteínas acumuladas en el edema pulmonar de estos pacientes, entre ellas el sFasL, pueden estar oxidadas.

OBJETIVOS Y PLANTEAMIENTO EXPERIMENTAL

En el presente estudio, nuestros objetivos principales fueron identificar los determinantes estructurales intrínsecos que modulan la actividad del sFasL humano, así como determinar los factores que modifican la estructura y la bioactividad del sFasL humano en el espacio aéreo pulmonar de pacientes con LPA/SDRA. Para ello clonamos, expresamos y purificamos una variante larga (con la región stalk) y una variante corta (sin la región stalk) del sFasL humano. Para determinar la importancia de la región stalk en la actividad de esta proteína, desarrollamos un modelo animal en ratones en los que instilamos por vía intratraqueal una dosis de la forma larga o corta del sFasL humano. Para determinar si el LBA de pacientes con LPA/SDRA contiene oxidantes que modifican la estructura y la actividad del sFasL humano, realizamos estudios *in vitro* en los que usamos fluido de LBA de pacientes con LPA/SDRA, así como oxidantes derivados de mieloperoxidasas. Finalmente, realizamos también estudios *in vitro* para determinar cuáles son los aminoácidos claves implicados en las alteraciones estructurales y funcionales del sFasL bajo condiciones oxidantes.

CONCLUSIONES

De los hallazgos encontrados en el presente estudio podemos extraer las siguientes conclusiones:

1. La forma soluble de Fas Ligando (sFasL) induce lesión en pulmones de ratón mediante la activación específica del receptor Fas.

2. La región stalk en el N-terminal es un determinante crítico para la actividad biológica del sFasL humano tanto en pulmones *in vivo* como en células Jurkat *in vitro*. La región stalk, sin embargo, no afecta el grado de agregación de sFasL. La región stalk del sFasL puede ser segmentada in vitro por la MMP-7, lo cual resulta en una disminución de su bioactividad.

3. El fluido del LBA de pacientes con SDRA contiene factores que:

   a. Incrementan la actividad del sFasL.
   b. Aumentan la agregación del sFasL mediante la formación de enlaces covalentes intermoleculares, lo cual le confiere una mayor estabilidad y mayor resistencia a la degradación.

4. Los residuos de metionina del sFasL humano son determinantes críticos de la actividad de esta proteína bajo condiciones pro-oxidantes. En particular, la exposición a HOCl, un oxidante derivado de la mieloperoxidasa, modifica la bioactividad del sFasL y promueve su agregación por mecanismos derivados de la oxidación de las metioninas presentes en esta proteína.

En base a estos resultados, nosotros proponemos que la actividad biológica del sFasL en fluidos fisiológicos podría estar determinada por la proporción relativa de sus variantes estructurales. Los oxidantes derivados de neutrófilos presentes en el edema pulmonar de pacientes con LPA/SDRA podrían modificar la estructura y la actividad del sFasL en los pulmones de estos pacientes. Un mejor conocimiento de los mecanismos moleculares que modulan la actividad del sFasL podría resultar en estrategias terapéuticas dirigidas a modificar la actividad biológica del sFasL *in vivo*. En este sentido, la región stalk o las metioninas podrían ser dianas terapéuticas para modular la actividad del sFasL al inicio del desarrollo de la LPA/SDRA.

4. – INTRODUCTION

4.1. GENERALITIES OF ACUTE LUNG INJURY

4.1.1. DEFINITION OF ACUTE LUNG INJURY AND ACUTE RESPIRATORY DISTRESS SYNDROME

Acute lung injury (ALI) and acute respiratory distress syndrome (ARDS) are common problems in the ICU that affect both medical and surgical patients. ALI/ARDS is a major cause of acute respiratory failure with high morbidity and mortality in critically ill patients. ALI and ARDS are the same clinical disorder but they are distinguished by the severity of hypoxemia. ALI/ARDS is characterized by widespread inflammation and non-hydrostatic pulmonary edema resulting from alveolar-capillary damage caused by multiple factors. Acute lung injury is defined by physiologic and radiographic criteria in which widespread damage to cells and structures of the alveolar capillary membrane occurs within hours to days of a predisposing insult. The first description of acute respiratory distress syndrome appeared in 1967, when Ashbaugh and colleagues described 12 patients with acute respiratory distress, cyanosis refractory to oxygen therapy, decreased lung compliance, and diffuse infiltrates evident on the chest radiograph [1]. Initially called the adult respiratory distress syndrome [2], this entity is now termed the acute respiratory distress syndrome, since it does occur in children. The first description of this illness paralleled the creation of Respiratory Intensive Care Units (ICU) and the treatment of acute respiratory failure with positive pressure.

Although the concept of ARDS was clear, more objective and validated parameters were needed to systematically identify all patients with this disease. In 1988, Murray and colleagues developed the Lung Injury Score to unify diagnostic criteria [3]. The score quantified the physiologic respiratory impairment through the use of a four-point lung-injury scoring system that was based on the level of positive end-expiratory pressure (PEEP), the ratio of the partial pressure of arterial oxygen to the fraction of inspired oxygen, the static lung compliance, and the degree of infiltration evident on chest radiographs. Other factors included in the assessment were the inciting clinical disorder, and the presence or absence of nonpulmonary organ dysfunction. Although this score helped quantify the severity of lung injury, it had limited clinical utility since it did not predict outcomes during the first 24 to 72 hours after the onset of the acute respiratory distress syndrome [4, 5]. By four to seven days after the onset of the

syndrome, scores of 2.5 or higher predicted a complicated course with the need for prolonged mechanical ventilation [6].

In 1994, a new definition was recommended by the American–European Consensus Conference (AECC) Committee (Table 4.1) [7]. This new definition is currently used in most intensive care units and has important advantages. It recognizes that acute lung injury is a progressive disorder with different degrees of severity. Patients with less severe hypoxemia (as defined by a ratio of the partial pressure of arterial oxygen to the fraction of inspired oxygen of 300 or less) are considered to have acute lung injury (ALI), and those with more severe hypoxemia (as defined by a ratio of 200 or less) are considered to have the acute respiratory distress syndrome (ARDS) [4, 5].

Table 4.1

Consensus Definition of ALI/ARDS by the American–European Consensus Conference (AECC) ***1994, Bernard et al*** [8]
Acute onset of respiratory failure
Bilateral infiltrates on chest radiograph
Absence of left atrial hypertension (pulmonary artery occlusive pressure* [PAOP] ≤ 18 mm Hg) or no clinical evidence of left atrial hypertension
Hypoxemia, defined as PaO_2/FIO_2 ≤ 300 (ALI) or ≤ 200 (ARDS) mmHg †

* If available. There is no consensus mandating pulmonary artery catheter insertion in suspected ALI/ARDS unless otherwise clinically indicated.

† PaO_2 = partial pressure of arterial oxygen (in mm Hg),
FIO_2 = fraction of inspired oxygen (in decimal fraction (eg, 0.5)).

This new definition permits earlier identification of patients with this disorder, although distinguishing ALI from ARDS is arbitrary, because hypoxemia correlates poorly with lung pathology and clinical course. However, the definition is simple and easy to use, especially in clinical trials. It has some limitations because it does not include important factors that influence outcome, such as the underlying cause, and whether other organ systems are affected [9-11]. In addition, the criterion for the presence of bilateral infiltrates on chest radiography is not sufficiently specific to be applied uniformly by experienced clinician [12, 13]. The PaO_2/FiO_2 relationship cannot be precise,

because it can vary when the FiO_2 and/or other parameters of ventilatory support are modified [14, 15]. Esteban et al. compared these accepted clinical criteria with post-mortem examinations of the lungs of patients with ARDS, and found that the definition of ARDS given by the American–European Consensus Conference Committee had a moderate ability to identify patients with ARDS defined by pathology (sensitivity = 75% y specificity = 84%) [16].

Nevertheless, the widespread acceptance of the 1994 consensus definition facilitates the early identification of patients with ARDS in the clinical setting [7], particularly those who would benefit from mechanical ventilation with low tidal volumes [17], and it also permits a better standardization of clinical research and trials.

4.1.2. EPIDEMIOLOGY AND SOCIAL RELEVANCE OF ACUTE LUNG INJURY

4.1.2.1. Incidence

ALI/ARDS is a cause of acute respiratory failure that develops in patients of all ages from a variety of clinical disorders, including pneumonia (bacterial, viral, and fungal), sepsis (pulmonary and nonpulmonary), aspiration of gastric and oropharyngeal contents, major trauma, and several other clinical disorders including severe acute pancreatitis, drug over dose, and transfusion of blood products [18, 19]. Most patients require assisted ventilation with positive pressure.

The exact incidence of ALI/ARDS has been difficult to estimate because of the absence of a common definition, and the wide variety of causes and coexisting diseases. The National Institutes of Health (NIH) first estimated the incidence at 75 per 100,000 persons in 1977 [20]. Since then, a number of studies reported lower incidences [21]. For example, in the late 1980s and early 1990s, studies found relatively low ARDS incidence rates (1.5 to 8.3 cases per 100,000 population per year) [22-24]. However, from 1997 onward, studies using the more liberal AECC criteria found higher incidence rates (4.9 to 75 cases per 100,000 population per year) [25-28]. In particular, a study by the National Heart, Lung and Blood Institute-sponsored ARDS Network of 20 hospitals estimated that the incidence of ALI/ARDS could be as high as 64 cases per 100,000 persons per year; not far off from the original NIH estimate. This dataset has the

advantage of being prospectively collected from a large number of academic medical centers [29].

4.1.2.2. Mortality

Until recently, the fatality rate of ARDS was considered to exceed 50% [5, 30, 31]. The majority of deaths are caused by sepsis or multiorgan dysfunction rather than primary respiratory failure [4, 5, 9, 10, 28, 32], however, the therapeutic success of ventilation with low tidal volumes indicates that in some cases death is directly related to lung injury [30, 33]. Recent epidemiologic data indicate that ARDS may account for 73,000 deaths per year in a country the size of the U.S. [25]. Although there is evidence that mortality in patients with ALI/ARDS may have declined over the last 10 to 15 years, it remains high (30-40%), and it is an important cause of pulmonary and nonpulmonary morbidity in patients who leave the hospital [34-37]. Possible reasons for the decrease in mortality include more effective treatments for sepsis, changes in the method of mechanical ventilation, and improvement in the supportive care of critically ill patients. This decrease in mortality suggests that there are additional opportunities to improve outcomes in ALI/ARDS if we can increase our knowledge at the basic, translational, and clinical levels.

4.1.3. ETIOLOGY AND RISK FACTORS

ALI/ARDS is caused by a multitude of disorders that directly or indirectly injure the lungs (Table 4.2) [38]. The most common cause of direct lung injury is pneumonia which may be of bacterial, viral or fungal origin. Sepsis is the most common cause of indirect lung injury with an overall risk of progression to ALI or ARDS of 30% to 40%[38, 39 40, 41]. Severe trauma with shock and multiple transfusions are also important predisposing conditions to indirect lung injury [42-47]. Multiple clinical conditions are considered to be risk factors for the development of ALI/ARDS. The most frequently encountered clinical risk factors include severe trauma (particularly lung contusion, fat emboli from long bone fractures), acute pancreatitis, aspiration of gastric contents, multiple transfusions of blood products, especially of fresh-frozen plasma, high net fluid balance [48], cardiopulmonary bypass, shock and the systemic inflammatory response syndrome (SIRS), near-drowning, burns and disseminated intravascular coagulation (DIC) [19, 38, 45,

[49]. When more than one of these clinical risk factors are present, the likelihood of ARDS is greater than just the sum of the collective risk factors [40]. Physiological risk factors for ARDS include metabolic acidosis, hypoalbuminemia and low respiratory compliance [50-52].

The risk of developing acute lung injury also depends on patients' characteristics, such as pre-existing chronic illness, particularly chronic lung diseases [51], postoperative state, alcoholism [53], malnutrition, advanced age, morbid obesity and cigarette smoking[54, 55]. To some extent, every patient in the ICU is at risk for the development ALI/ARDS.

Table 4.2.

Clinical Conditions Associated with Development of Acute Respiratory Distress Syndrome

Direct Lung Injury	**Indirect Lung Injury**
Common causes	***Common causes***
Pneumonia	Sepsis
Aspiration of gastric contents	Severe trauma
Less common causes	***Less common causes***
Pulmonary contusion	Acute pancreatitis
Fat embolism	Cardiopulmonary bypass
Near-drowning	Massive blood transfusion (>15 U) (TRALI)*
Inhalation injury	Drug overdose (eg, aspirin, cocaine, opioids, phenothiazines, tricyclics)
Reperfusion pulmonary edema post lung transplantation or pulmonary embolectomy	Disseminated intravascular coagulation (DIC)
Lung transplantation	Bone marrow transplantation
Diffuse alveolar hemorrhage	Burns
	Radiographic contrast (rare)
	Stroke or seizure (neurogenic pulmonary edema)
	Organ transplantation (specially when transplant rejection occurs)

Adapted from Ware LB and Matthay MA: The acute respiratory distress syndrome. *N Engl J Med* 2000; 342(18):1334–1349.

*TRALI= Transfusion related acute lung injury

It is not clear whether the distinction between direct (pulmonary) and indirect (extrapulmonary) lung injury is clinically useful. Some investigators have demonstrated reduced respiratory system compliance in patients with ARDS due to direct pulmonary

injury as compared with the indirect causes, although total respiratory system compliance (including the chest wall) is similar. Patients with direct lung injury may be more likely to have improved lung mechanics with application of positive end-expiratory pressure (PEEP). However, in the largest cohort of patients studied there was no difference in mortality between those with direct and indirect causes of lung injury [56 57, 58] The outcomes are similar in both categories if age, underlying chronic illnesses, and severity of non-pulmonary illness and gas-exchange abnormalities are controlled for [57, 59, 60]. Regardless of the underlying cause of ALI/ARDS most patients with ALI/ARDS appear to have a systemic inflammatory response syndrome (SIRS), which can lead to multiorgan dysfunction [61].

The identification of risk factors to develop ALI/ARDS is very important to initiate early treatments and to prevent the progression of lung damage in patients with high risk. In recent years, new risk factors have been described, for example the infection of the virus of the Severe Acute Respiratory Syndrome (SARS) [62] and the ventilator-inducing lung injury (VILI) when conventional parameters of ventilation are used (high tidal volume, peak pressure > 30 cmH_2O and PEEP > 5 cmH_2O) [33, 63]. Development of new-onset ARDS is a relatively common complication (152 of 798 patients) in those receiving mechanical ventilation for ≥ 48 h in the ICU [50]. High airway pressure and tidal volume were the most important ventilator-associated risk factors for the development of new ARDS [50].

Recent data now suggest the possibility of a genetic predisposition that influences susceptibility to ALI/ARDS. In sepsis, trauma, and other triggering conditions, only a subset of patients develops ALI/ARDS even among those in whom the pathologic stimuli are apparently equivalent, suggesting that there are genetic features that may influence its onset. There is suggestive evidence for genetic variations in ALI/ ARDS and in predisposing conditions such as sepsis. Polymorphisms in genes related to inflammatory markers (tumor necrosis factor alpha, surfactant proteins, interleukin-6, IL-1RA), angiotensin converting enzyme, and pathogen receptors (CD14, toll-like receptors) appear to correlate with incidence and/or outcome of sepsis [64, 65 66, 67] and/or ALI/ARDS [68, 69]. These observations were based on case-control association studies. Family-based association studies and case-controlled association studies may be beneficial to explore in well-defined subgroups of patients with ALI/ARDS.

4.1.4. CLINICAL MANIFESTATIONS AND DIAGNOSIS

In the majority of patients, the initial diagnosis of ALI/ARDS is made following clinical criteria because currently there is no laboratory test that determines the presence or absence of ALI/ARDS. Once diagnosed, testing to identify the underlying cause is required. Symptoms and signs are nonspecific and typically develop within 24 to 48 h of initial injury or illness. Dyspnea is the primary symptom, occasionally accompanied by cough or chest pain. Signs invariably include tachypnea and tachycardia. Accessory muscle use, cyanotic skin, and abnormal breath sounds (crackles, rhonchi, and/or wheezes) may be present. ALI/ARDS is suspected when dyspnea and respiratory insufficiency rapidly develop in settings that predispose to ARDS, and requires the acute onset of bilateral radiographic infiltrates. Although the presence of bilateral infiltrates is a hallmark of ALI/ARDS, the radiographic findings are not specific. A similar clinical presentation can result from acute heart failure and from bilateral pulmonary infections. All patients should have arterial blood gases (ABGs) and a chest x-ray. The diagnosis is confirmed by demonstration of hypoxemia and widespread chest x-ray infiltrates in the absence of clinical or manometric signs of left atrial hypertension. According to the AECC criteria, the pulmonary artery wedge pressure (PAWP) should be less than or equal to 18 mm Hg if measured (a PAOP > 20 mm Hg suggests heart failure or hypervolemia) but this measurement is not foolproof because ALI/ARDS can be complicated by volume overload, severe hypoproteinemia or heart failure with concomitant increases in the left atrial pressure [7, 12, 70, 71].

Invasive techniques for diagnosis have limited clinical utility and the benefits rarely outweigh the risk. In the past, open lung biopsy was obtained more frequently for diagnosis. Interestingly, the degree of histologic abnormality on lung biopsy did not correlate with ultimate outcome as measured by pulmonary function. Open or thoracoscopic lung biopsy may still be useful in some cases where the diagnosis is uncertain and the underlying cause in not apparent. Occasionally unsuspected diagnoses requiring specific therapy can be made such as miliary tuberculosis, pulmonary blastomycosis, eosinophilic pneumonia, or bronchiolitis obliterans organizing pneumonia [72, 73]. Bronchoscopy also has a limited role in diagnosis and may be most useful in the immunocompromised host. Bronchoalveolar lavage (BAL) for cultures and cytologic examination can identify the cause of pneumonia and is particularly useful in the diagnosis of opportunistic infections [74]. Bronchoalveolar Lavage fluid (BAL) usually has a predominance of neutrophils and there may be

evidence of diffuse alveolar hemorrhage. When the cause is still uncertain, cytologic examination of BAL fluid can be used to confirm the presence of diffuse alveolar damage, malignancy or other disorders[75, 76-78].

4.1.4.1. Early phase of ALI/ARDS

Clinically, the acute phase is manifested by the acute onset of radiographic infiltrates consistent with pulmonary edema, hypoxemia and increased work of breathing. The chest x-ray shows bilateral infiltrates (by definition), symmetrical or asymmetrical, that can be patchy or diffuse, and fluffy or dense. Chest computed tomographic (CT) imaging, although rarely of use clinically, has been employed as an investigative tool to better define the nature of the infiltrates in patients with ALI/ARDS. The distribution of infiltrates by CT is very patchy; areas of alveolar filling and consolidation occur predominantly in dependent lung fields (dorsal caudal in supine patients) with interlobular septal thickening, and often small pleural effusions [79, 80, 81]. The nondependent regions can appear relatively spared. Even areas that appear spared in radiographic images may have substantial inflammation when sampled using bronchoalveolar lavage [82].

The hypoxemia that characterizes early ALI/ARDS is usually relatively refractory to supplemental oxygen because the pathophysiology involves shunting of blood through areas of unventilated lung. The increased work of breathing in the acute phase of ALI/ARDS is due to decreased lung compliance as a result of alveolar and interstitial edema combined with increased airflow resistance [81]. The combination of hypoxemia and increased work of breathing usually necessitates endotracheal intubation and mechanical ventilation, although occasionally patients can be managed with noninvasive ventilation.

4.1.4.2. Late fibroproliferative phase of ALI/ARDS

In most patients, ALI/ARDS will resolve after the acute phase. However, in others a fibrosing alveolitis becomes clinically apparent after 7 to 10 days, although evidence of deposition of extracellular matrix has been identified in alveolar lining fluid from patients as early as the first day after intubation [83, 84]. Radiographically, reticulonodular and ground-glass opacities with honeycombing and/or bullae are apparent on chest x-ray

and CT consistent with the evolving fibrosis. Histologically, pulmonary edema and neutrophilic inflammation are less prominent. A severe fibroproliferative process fills the airspaces with granulation tissue that contains extracellular matrix rich in collagen and fibrin as well as new blood vessels and proliferating mesenchymal cells [81, 85].

Clinically the late fibroproliferative phase of ALI/ARDS is characterized by continued need for mechanical ventilation often with persistently high levels of PEEP and FiO2. The lung compliance may fall even further and pulmonary dead space is elevated [81, 85]. Pulmonary hypertension may develop due to obliteration of the pulmonary capillary bed and right ventricular failure may occur [86]. A prolonged stage of fibrosing alveolitis can make weaning from the mechanical ventilation difficult.

4.1.4.3. Resolution of ALI/ARDS

Lung biopsy samples from ALI/ARDS survivors typically show normal or near-normal lung histology. For a complete histological resolution of ALI/ARDS, a variety of processes must be reversed. Alveolar edema is actively reabsorbed by the vectorial transport of sodium and chloride from the distal airway and alveolar spaces into the lung interstitium. Water is passively absorbed along the osmotic gradient, through water channels, the aquaporins. The majority of patients with early ALI/ARDS have impaired alveolar transport [87-91]. Faster rates of alveolar epithelial fluid transport are associated with a better outcome [92]. Soluble and insoluble protein must also be cleared by lymphatics. Insoluble protein is most likely cleared by macrophage phagocytosis or alveolar epithelial cell endocytosis and transcytosis [93, 94].

The denuded alveolar epithelium must be repaired [91]. The alveolar epithelial type II cell serves as the progenitor cells for repopulating the alveolar epithelium. Alveolar epithelial type II cells proliferate migrate and differentiate to reconstitute a tight alveolar epithelial type I cell barrier[87 38]. The inflammatory cell infiltrate must also resolve, but here the mechanisms are less clear. Resolution of neutrophil inflammation may be predominant via neutrophil apoptosis and phagocytosis by macrophages. However, neutrophil apoptosis is impaired in the lungs of patients with ALI/ARDS [95, 96]. The resolution of fibrosis is also unclear. In patients with advanced fibrosis, resolution takes place over many months as pulmonary abnormalities improve [97, 98].

4.1.5. HISTOLOGICAL FEATURES OF ALI/ARDS

ALI/ARDS is a progressive syndrome characterized by distinct stages: exudative (or acute), and fibroproliferative phases, which have different histopathological manifestations. The course of each phase and the overall disease progression is variable. The exudative phase includes diffuse alveolar damage with alveolar flooding by protein-rich edema fluid, neutrophil influx and hemorrhage into the alveolar space, loss of alveolar epithelial cells, deposition of hyaline membranes on the denudated basement membrane, and formation of microthrombi [99] [38]. Alveolar flooding occurs as a result of injury to the alveolar-capillary barrier, which is formed by two separate cell layers: the microvascular endothelium and the alveolar epithelium. Ultrastructural studies reveal a vacuolization and swelling of the vascular endothelium [38]. This endothelial damage is associated to a procoagulant tendency responsible for the formation of capillary thrombosis [100, 101]. Despite this widespread endothelial damage, the hallmark of this syndrome is the disruption of the alveolar epithelium, which is a major determinant of the hypoxemia and altered lung mechanics that characterize early ALI/ARDS [87]. Damage of the epithelial barrier exacerbates the tendency for alveolar flooding, and delays recovery by impairing fluid clearance. Type I alveolar cells are irreversibly damaged and the denuded space is replaced by the deposition of proteins, fibrin, and cellular debris, producing hyaline membranes, while injury to the surfactant-producing type II cells contributes to alveolar collapse [91, 102-107]. The alveolar flooding in ALI/ARDS is characteristically with a protein-rich edema fluid due to the increased permeability of the alveolar capillary barrier, in contrast to the low protein pulmonary edema that results from hydrostatic causes such as congestive heart failure or acute myocardial infarction [99, 108, 109].

Although ALI/ARDS may resolve completely in some patients after the acute phase, in others it progresses to a fibroproliferative phase [85, 109, 110]. It is characterized by reepithelialization of the epithelial barrier by the proliferation of the alveolar type II cells. In the alveolar wall, fibroblasts also proliferate and migrate through the basement membrane into the fibrinous intra-alveolar exudate. In some patients, this progresses to an irreversible fibrosis of the lung involving collagen deposition in alveolar, vascular, and interstitial beds[111-113, 114].

4.1.6. TREATMENT AND CLINICAL COMPLICATIONS

Improvement in the supportive care of patients with ALI/ARDS, particularly the use of a lung protective ventilatory strategy, has contributed to reducing the mortality rate. However, despite numerous randomized controlled clinical trials, there is no specific treatment for ALI/ARDS, aside from reducing tidal volume and using a conservative fluid strategy.

4.1.6.1. Treatment of predisposing factors

A search for the underlying cause of ALI/ARDS should be undertaken. Appropriate treatment for any precipitating infection such as pneumonia or sepsis is critical to enhance the chance of survival. Abdominal infection should be treated promptly with antimicrobial agents or surgery.

4.1.6.2. Basic management

4.1.6.2.1. Prevention of common complications

During the course of ALI/ARDS, the patients can undergo multiple complications, most of which are preventable. Common complications include deep venous thrombosis and pulmonary embolism, stress-related gastrointestinal hemorrhage, ventilator associated pneumonia and nosocomial infections, metabolic abnormalities, polyneuropathy and malnutrition. Anticipation and prevention of these complications is vitally important [32, 49]. Prevention of gastrointestinal bleeding and thromboembolism is also very important [115].

4.1.6.2.2. Nutrition

Many lines of evidence favor the provision of adequate nutrition in critically ill patients. The enteral route is preferred to the parenteral route and is associated with less infectious complications. Enteral feeding also has other beneficial effects. The use of enteral nutrition is also important to prevent stress-related gastrointestinal bleeding and to maintain the normal barrier function of the gastrointestinal mucosa. Loss of the gastrointestinal barrier function has been associated with the translocation of bacteria

and/or toxins into the mesenteric lymph nodes and portal circulation which perpetuates the injury process [116]. In normal volunteers, administration of parenteral nutrition with bowel rest increased circulating levels of tumor necrosis factor-alpha, glucagon, and epinephrine and increased febrile responses compared with volunteers who received enteral nutrition [117-119].

The goal of the nutritional support includes providing adequate nutrients and the treatment of any deficiencies in micronutrients or macronutrients. Whether a particular dietary composition could be beneficial in patients with ALI/ARDS is unclear. In an attempt to improve the immune response, a diet containing a combination of omega-3 fatty acids, ribonucleotides, arginine and glutamine has been used. A meta-analysis of these trials suggested a beneficial effect on infection rate but not overall mortality. One randomized controlled trial in patients with ALI/ARDS has tested a diet rich in fish oil, gamma-linoleic acid and antioxidants; this diet was associated with shorter duration of mechanical ventilation and fewer organ failures, but no difference in mortality was observed. Overall, there is still no compelling evidence to support the use of anything other than standard enteral nutritional support with avoidance of overfeeding, in patients with ALI/ARDS to maintain the proper level of immune function and prevent malnutrition [116-118, 120].

4.1.6.3. Fluid and hemodynamic management

Controversy also surrounds the management of volume status and hemodynamics in patients with ALI/ARDS. A theoretical case can be made for aggressive diuresis, in an effort to reduce the formation of pulmonary edema. Indeed, in experimental lung injury, lower left atrial pressures are associated with less formation of pulmonary edema. Several studies have reported increase survival associated with low pulmonary capillary occlusion pressure in the setting of ALI/ARDS [121-123]. However, reduction in intravascular volume can have adverse effects on cardiac output and tissue perfusion, factors that can contribute to multiple organ failure. This is a legitimate concern, because mortality in ALI/ARDS is usually from nonpulmonary causes including other organ failures. This concern led to additional studies from the ARDS Network to evaluate liberal versus conservative fluid replacement strategy (FACTT trial) [124]. The liberal fluid management arm had an average net again of almost 7 L over the first 7 days after the initial 24 hr of volume resuscitation, whereas the conservative fluid

management group averaged a loss of 136 mL over the same period of time. The use of a conservative fluid management strategy was associated with a significant improvement in oxygenation index and lung injury score and increased the number of ventilator-free days compared with the more liberal strategy. There was no difference in the development of shock or the need for renal replacement therapy between the two fluid management strategies [125]. In addition, this trial also evaluated the utility and safety of using a pulmonary artery (PA) catheter to guide the volume replacement versus a central venous catheter in 1000 patients with established ALI. There was no difference in the fluid replacement but the pulmonary artery group did have twice as many catheter-related complications, predominantly in the form of arrhythmias, compared with the central venous catheter group [126]. In the study by Marty et al., human serum albumin was used to support vascular volume while diuresis was simultaneously accomplished using furosemide to reduce lung edema. In this pilot randomized clinical trial (n-37), such treatment produced transient improvements in oxygenation without compromising the circulation, although more studies are needed to evaluate the ultimate outcome [127]. Besides the controversies, a reasonable strategy is to achieve the lowest intravascular volume that maintains adequate tissue perfusion as measured by urine output or other organ perfusion and metabolic acid-base status. If organ perfusion cannot be maintained in the setting of adequate intravascular volume, then administration of vasopressors and/or inotropes should be used to restore end-organ perfusion [128].

4.1.6.4. Mechanical ventilation

Over the past decade there has been increasing recognition that the ventilatory support strategy used in the management of patients with ALI/ARDS may produce or augment lung injury and/or impair the healing process [129]. These fears initially arose from the results of experimental animal studies that demonstrated clinical, physiological, and histological evidence of lung injury, similar to that observed in patients with ARDS, when the animals were ventilated with large tidal volumes or were given high inflation pressures. The alveolar overdistension produced by these ventilatory modes was felt to be the critical element in production of the lung injury [130]. Lung injury could result form the administration of large volumes or the administration of positive or negative pressure breaths (termed volutrauma). Other mechanisms that could potentially result in lung injury were the repetitive recruitment-derecruitment of distal airways and alveoli

(termed atelectotrauma) and/or the disruption of alveoli resulting in translocation of organisms or air emboli [131-134]. Alveolar overdistention can also give rise to systemic inflammatory molecules that may contribute to the SIRS response and drive the development of MODS/MOF [135-137]. This has been termed biotrauma. A multicenter trial of low versus large tidal volume ventilation in patients with ALI/ARDS demonstrated increased proinflammatory cytokines in the serum and bronchoalveolar lavage fluid of patients ventilated with large traditional tidal volumes, supporting the concept of biotrauma from alveolar overdistention [135, 138-141]. Interestingly, the lung injury produced by high inflation pressure or large tidal volumes in these experimental models could be ameliorated by the addition of therapeutic amounts of PEEP [142].

Although historically a tidal volume of 12 to 15 mL/kg was recommended in patients with ALI/ARDS, it is now clear that a low tidal volume reduces mortality. The NIH ARDS Network published the findings of their first randomized, controlled, multicenter clinical trial in 861 patients [143]. The rationale for this trial was the growing clinical and experimental evidence suggesting that mechanical ventilation with high tidal volumes and high plateau pressures might be harmful to the injured lung. The trial was designed to compare a low tidal volume ventilatory strategy (6 ml/kg predicted body weight, plateau pressure less than 30 cm H2O) with a higher tidal volume (12 mL/kg predicted body weight, plateau pressure less than 50 cm H2O). The in-hospital mortality rate was 40% in the 12 mL/kg group and 31% in the 6 mL/kg group, a 22% reduction (p=0.007). This was the first clinical trial to demonstrate a mortality benefit of this lung protective ventilatory strategy. A significant difference occurred in the length of hospital stay between the low and high tidal volume groups. The use of lower tidal volumes typically results in a controlled hypoventilation or permissive hypercapnia and can lead to hypercapnic acidosis. A rise in the respiratory rate is usually sufficient to compensate for the decreased tidal volume; a rate as high as 35 breaths/min was used in the clinical trial [33].

Some experimental animal models of lung injury suggest that hypercapnic acidosis may be beneficial to the lung and produce less lung injury as measured by extravascular lung water [144]. The PEEP is also a major component of the ventilatory support strategy for the patient with ALI/ARDS. In fact, PEEP may have a direct therapeutic role in the prevention of ventilator-induced lung injury, as noted in some of the experimental models of ventilator-inducing lung injury (VILI). The use of PEEP has

assisted in the recruitment of atelectatic lung units, prevented recruitment-derecruitment, increased the functional residual capacity (FRC), decreased the shunt fraction, and allowed for a reduction to a less toxic FiO_2, while still maintaining adequate oxygenation saturation and tissue oxygen delivery [142]. All these protective ventilatory strategies were associated with decreased serum cytokine and chemokine levels [145], decreased levels of organ dysfunction, and decreased mortality in patients with ARDS [146].

During mechanical ventilation, some patients have significant dyssynchrony with the ventilator. Increasing the inspiratory flow rate and, if necessary, the level of sedation is usually sufficient to manage these problems. Patients with severe refractory hypoxemia not responding even to a FiO_2 of 1.0 and a PEEP level of 24 cm H_2O or more will require deeper sedation and neuromuscular blockade, although the latter must be considered a rescue therapy as it can increase the risk of polyneuropathy and myopathy [147].

4.1.6.5. Non-invasive ventilation

Non-invasive positive-pressure ventilation delivered by nasal or full facemask has been successful in avoidance of intubation in patients with exacerbation of chronic obstructive pulmonary disease (COPD). The role for non-invasive ventilation in ALI/ARDS is less clear. In one large had a failure rate of 30% for 354 patients who were not already intubated, and 51% for the patients with ARDS [148]. It seems likely that the majority of patients with ALI/ARDS will still require invasive mechanical ventilation.

4.1.6.6. Inhaled Nitric Oxide

Inhaled nitric oxide is a potent endothelium derived relaxing factor of the bronchial and vascular smooth muscle reported to decrease platelet adherence and have anti-inflammatory properties. It has been shown to increase oxygenation by improving blood flow to well-ventilated areas and decrease intrapulmonary shunting in the lung. A reduction in pulmonary arterial pressure and pulmonary vascular resistance also occurs, and has minimal systemic effects [149]. Phase II and III trials with inhaled nitric oxide alone in patients with ARDS showed mild and transient improvement in

oxygenation but failed to demonstrate improvement in mortality or reduction in ventilator days [150, 151].

4.1.6.7. Surfactant replacement therapy

Surfactant abnormalities are present in patients with ALI/ARDS related to decreased production of surfactant products, inactivation by alveolar proteins and proteolytic enzymes, and dilution by the alveolar fluid. Anzueto et al. reported that the surfactant replacement did not result in any difference in hemodynamic function, oxygenation, mechanical ventilation-free days and survival in 725 patients with ARDS caused by sepsis who were prospectively randomized into surfactant versus placebo groups [152]. It was suggested that the absence of surfactant proteins might account for the lack of efficacy. Trials are continuing evaluating surfactant proteins. A small clinical trial evaluating recombinant surfactant protein C replacement in patients with ARDS noted an improvement in oxygenation but no survival benefit [153].

4.1.6.8. Enhance edema clearance

Accumulated fluid in the alveolus could potentially worsen the gas exchange, as well as produce adverse pulmonary mechanics with an increased work of breathing. Recent strategies have been designed to improve the edema fluid clearance either using aquaporins or increasing the activity of the Na/K pump [154-161]. Studies in rats demonstrated that a β_2-adrenergic agonist can enhance the clearance of lung edema and improve oxygenation in the resolution phase of hydrostatic pulmonary edema [155]. In isolated perfused rat lungs, there was also a significant increase in fluid clearance with administration of isoproterenol or dopamine. In a recent study of the potential therapeutic value of β_2-adrenergic agonist therapy for the treatment of acid induced lung injury in rats, they found that salmeterol, a β_2-adrenergic agonist given via the airspaces, reduced lung edema by both attenuating lung vascular injury and upregulating the clearance of alveolar edema fluid [105]. From a practical perspective, it seems that the potential benefit of β_2-adrenergic therapy would be achieved with 3 to 4 days of treatment, a time period during which a significant downregulation to the aerosolized agonist seems unlikely. From a clinical perspective, aerosolized β_2-agonist therapy has been shown to reduce the incidence of high-altitude pulmonary edema in subjects at-risk for developing lung injury. A small clinical trial in the United Kingdom

has found that intravenous salbutamol, a β_2-agonist, reduces extravascular lung water in patients with acute lung injury. It may be reasonable to test the therapeutic value of aerosolized β_2-adrenergic agonist therapy for patients with acute lung injury, particularly because one study demonstrated that therapeutic levels of a commonly used β_2-agonist can be achieved in the pulmonary edema fluid in ventilated patients with standard aerosolization procedures [162]. The evaluated the issue of intravenous beta-agonist (salbutamol) in patients with ALI and demonstrated a significant decrease in the extravascular lung water at day 7 [163]. The salbutamol-treated group also had lower end-inspiratory plateau pressures. This group had more supraventricular arrhythmias [163]. Further investigation is necessary to determine the benefit of edema clearance strategies in the management of ALI patients.

4.1.6.9. Corticosteroids

A variety of treatment strategies have been investigated in large randomized trials with a predominant focus on anti-inflammatory strategies, particularly using glucocorticoids. However, glucocorticoids had no benefit when they given before the onset of the disease or early in its course [164, 165]. More recently, glucocorticoids have been used to treat the later, fibrosing-alveolitis phase of the disease. The use of corticosteroid rescue for the patients with persistent ARDS or in the fibroproliferative phase of ARDS was evaluated by the NIH-sponsored ARDS network. They studied 180 patients with ARDS greater than or equal to 7 days into a placebo-controlled trial of methylprednisolone versus placebo. The use of methylprednisolone was associated with early improvement in mortality, PaO_2/FiO_2 ratio, blood pressure, ventilator and ICU-free days. An increase in white blood cell count and glucose level related to steroid administration occurred with a decrease in body temperature. The steroid treatment was not associated with an increase in serious infection. In fact, there was more pneumonia and septic shock seen in the placebo group than in the steroid-treated group. However, no difference was measured in the 60-day mortality or in the 180-day outcome between two groups [166]. For now, the use of rescue steroids remains controversial. A short course of corticosteroids could be used as rescue therapy in patients with severe disease that is not resolving.

4.1.6.10. Anti-inflammatory and anticoagulant factors

Ibuprofen, a non-steroidal anti-inflammatory drug, failed to improve the outcome of critically ill patients with sepsis or septic shock and failed to prevent the development of ARDS in a prospective, randomized, placebo-controlled, multicenter clinical trial [167]

There is evidence that, like sepsis, ALI/ARDS is a procoagulant, antifibrinolytic state. A number of clinical trials evaluated the potential benefit of anticoagulant agents such as antithrombin III, activated protein C, and the tissue factor pathway inhibitor (TFPI) to prevent or treat the microthrombosis of the microcirculatory bed that occurs in sepsis and could result in ALI/ARDS as an early manifestation of MODS/MOF. To date, this strategy has not been found to decrease the development of or improve the outcome of ALI/ARDS [168-170].

Toxic oxygen radicals from the activated inflammatory cells are increased in the lungs of patients with ARDS. The abundant production of toxic oxygen radicals may overwhelm the ability of the endogenous oxygen radical scavengers, superoxide dismutase (SOD), catalase, and the glutathione reductase cycle. The administration of antioxidants such as N-acetylcysteine, procysteine, vitamine E, β-carotene, and vitamin C have been evaluated in the prevention and/or management of patients with ARDS. No survival benefit was seen associated with the administration of N-acetylcysteine or procysteine versus placebo in patients with ALI/ARDS [171]

Two published trials have demonstrated a significant reduction in the development of ARDS in surgical patients when ketoconazole, an imidazole thromboxane A2 synthetase inhibitor, was administered to an at-risk population of patients. When ketoconazole was used in patients with established ARDS, there was no benefit on survival [172]. More studies are needed to know the real effect of this drug on prevention of ALI/ARDS in at-risk patients [172].

4.1.6.11. Keratinocyte growth factor

Keratinocyte grow factor (KGF) is a potent epithelial mitogen produced and secreted by stromal cells. Epithelial cells express the KGF receptor and are the main target of this growth factor. KGF is an important mediator of epithelial-mesenchymal interactions

and epithelial repair in multiple organ systems, including the skin, intestine, and bladder. In animal models, pretreatment with KGF ameliorates experimentally induced acute lung injury [173, 174]. This protective effect was due to an improvement of the alveolar epithelial repair by increasing the proliferation and migration of alveolar type II, probably through stimulation of the epidermal growth factor receptor [175]. Also, KGF seems to increase in the clearance rate of alveolar fluid [176], induce antioxidants effects[177] and reduce lung endothelial injury [178].

4.1.6.12. Complications

Complications are common in any critically ill patient population. Support care and vigilance in both preventing and diagnosing common complications such as pulmonary embolism, acute myocardial infarction, gastrointestinal bleeding and nosocomial infection.

4.1.6.12.1. Barotrauma

Barotrauma occurs when air dissects out the airways or alveolar space into surrounding tissues, leading to pneumothorax, pneumomediastinum, pneumatocele or subcutaneous emphysema. The exact incidence of pulmonary barotrauma is unclear but appears to be declining. In the NIH ARDS Network trial, approximately 10% of patients developed some form of barotrauma regardless of whether they were in the 6 or 12 mL/kg tidal volume arm. Furthermore, PEEP level was the only factor that predicted the development of barotrauma in a multivariate analysis [33]. Pneumothorax can be life threatening particularly if it is under tension, and immediate diagnosis and tube thoracostomy is essential. Pneumomediastinum and subcutaneous emphysema can be painful but, other than analgesia, do not require specific therapy. Air embolus is a potentially fatal complication that has been reported occasionally in patients with ALI/ARDS and usually occurs in conjugation with other evidence of pulmonary barotrauma, many times simultaneously [179].

4.1.6.12.2. Nosocomial pneumonia

The estimate incidence of nosocomial pneumonia is 15 to 60% of patients. Autopsy studies of patients dying with ALI/ARDS show a high incidence of unexpected

pneumonia [61, 180, 181]. The adequacy and timeliness of initial empirical therapy are important determinants of outcome. Knowledge of local resistance patterns is crucial, and a high index of suspicion is required.

4.1.6.12.3. Multiple organ dysfunction syndrome

A frequent complication of an exaggerated proinflammatory state in the setting of sepsis, systemic inflammatory response syndrome (SIRS) and ARDS is the development of organ systemic dysfunction. This dysfunction may involve single or multiple organs. The dysfunction of two or more organs is considered as multiple organ dysfunction syndrome (MODS), and it is the most common cause of death in the noncoronary intensive care units. Organ dysfunction may result from the underlying cause of ALI/ARDS, such as sepsis, or occur independently, probably from an excessive systemic inflammatory response. Multiple combinations of direct lung injury, ischemic injury, circulatory humoral or inflammatory mediators, translocation of endotoxin and/or colonic bacteria, altered rheologic properties of the blood cells, or the iatrogenic effects of the therapy administered may interact in the eventual production of MODS [182-184].

4.1.6.12.4. Neuromuscular weakness

Patients with ALI/ARDS are at high risk for developing prolonged muscle weakness that persists after resolution of pulmonary infiltrates and can complicate weaning from mechanical ventilation and rehabilitation. One study suggests that neuromuscular abnormalities are persistent in many survivors of critical illness, even when studied up to 5 years after ICU discharge. Prolonged muscle weakness is more common in critically ill patients who are treated with glucocorticoids. In other studies, neuromuscular blockade has also been implicated [97, 185].

4.1.7. PROGNOSIS

Prior to the 1990s, mortality in clinical trials of patients with ALI/ARDS was 40% to 60%. The risk of in-hospital mortality was highest in those with sepsis (43%), intermediate in those with pneumonia (36%) or aspiration (37%) and lowest in those

with multiple trauma (11%). Reported mortality from ALI/ARDS appears to be gradually declining. The low tidal volume strategy was effective at reducing mortality across all causes of ALI/ARDS (31% in the 6 mL/kg tidal volume arm and 40% in the 12 mL/kg tidal volume arm). Factors that are associated independently with mortality from ALI/ARDS include age, septic and septic shock, acute physiology score, multiple organ failures, immunosuppression and chronic liver disease [4, 5, 10, 25]. Young trauma patients have the best outcomes [186]. Mortality is also associated with other underlying diseases such as HIV infection or cancer. Patients with a higher severity of illness score are more likely to develop ALI/ARDS and die of lung injury. Surprisingly, initial indexes of oxygenation and ventilation, including the PaO2/FiO2 ratio, PEEP and plateau pressure or gas exchange ($PaCO_2$ and VE40) and the lung injury score do not predict outcome [4, 5, 10]. However, the failure of pulmonary function to improve during the first week of treatment is a negative prognosis factor [6, 187]. An elevated dead space fraction has also been shown to be an independent risk for death [188]. In cases of ARDS, death is usually caused by progressive multisystem organ failure rather than respiratory deterioration [189].

Usually, survivors start to recover within two weeks of the onset of ARDS [189], however, they frequently have long-term functional disability. Interestingly, pulmonary function returns to normal or near normal in survivors. It has been reported in a one-year follow-up study of 109 survivors of ARDS, that lung volumes and spirometry returned to normal within 6 to 12 months, despite the severe injury to the lung [98, 190]. However, carbon monoxide diffusing capacity was persistently low at 12 months, largely due to muscle wasting and weakness rather than pulmonary function abnormalities. Residual impairment of pulmonary mechanisms may include mild restriction, obstruction, impairment of the diffusing capacity of carbon monoxide, or gas-exchange abnormalities with exercises, but these abnormalities are usually asymptomatic [191-193]. Severe disease and prolonged mechanical ventilation predict patients at highest risk for persistent pulmonary function alterations [98, 194]. Neuropsychologic testing may reveal significant deficits in patients who had more severe and protracted hypoxemia [195]. Neuropsychiatric problems and neuromuscular weakness are now known to occur frequently and are the major limitation for these patients to regain their daily activities [35, 97, 195, 196]. Patients who survived from ALI/ARDS have been reported to have both reduced health-related quality of life and reduced pulmonary disease-specific health-related quality of life [35, 98, 196].

4.1.8. FUTURE CONSIDERATIONS

During the past 30 years, there has been considerable progress in standardizing the evaluation and management of this disease worldwide. A better understanding of the pathophysiology has produced management strategies that have translated into evidence-based improvement in outcome. Experimental studies are being conducted to evaluate the role of epithelial growth factors and beta-adrenergic agonists in reducing lung injury and hastening repair. The growing knowledge of molecular biology and the elaborate mechanisms that govern an individual's response to injury, repair and cell death will likely have a major role in the management of patients with ALI/ARDS. Investigation into the role of gene mutations and gene polymorphisms has given insight into possible genetic susceptibility for the development and outcome of ARDS. Individuals with increased risk for ALI and ARDS development will probably be identified on the basis of their genetic profile. It is possible that in the future we may potentially modify the genetic makeup or the biologic response of a susceptible individual by inserting selected genes or modifying the transcription or function of various regulatory proteins [197]. Proteomic technologies have the potential to identify each protein expressed in isolated cells or in complex tissues such as injured lung and also to determine posttranslational modifications, protein-protein interactions, and other critical features that define phenotype and regulate cellular function. Proteomic approaches can provide physiologic relevance that is not inherent in measuring transcript levels and profiles in ALI/ARDS [198, 199]. Proteomic analysis of clinical samples may be useful in establishing molecular patterns that are characteristic of individual diseases or disease stages.

Another approach for the future treatment of ALI/ARDS could involve stem cells to replace dying lung cells. A similar strategy has been proposed for brain, heart, and liver diseases. The inflammatory response includes release of cells from bone marrow, such as mature and immature neutrophils and stem cells. Elegant bone marrow reconstitution studies have demonstrated that these stem cells have the potential to differentiate into endothelial, mesenchymal, and epithelial cells [200-202]. The proliferative potential of fully differentiated capillary endothelial cells and epithelial type I cells is very poor in the lungs. The potential for stem cells to help in the repair of injured lung tissue has not been explored and requires a better understanding of stem cell release, targeting, proliferation, and differentiation in lung tissue. Supplementing this release by intravenous infusion of donor bone marrow-derived stem cells might be useful in

ALI/ARDS, as the pulmonary microvasculature would be the first capillary bed that these cells would encounter, enhancing their entrapment and accumulation. Newly implanted endothelial cells may help restore the lung capillary network after ALI, although considerable work will be needed to develop approaches for delivery of viable cells to injured areas of the lung [203, 204].

As more strategies and drugs are developed, there is hope that control of this once fatal disease will be possible. For now, treating the inciting cause, avoiding ventilator-induced injury, managing fluids judiciously, and providing supportive care remain the cornerstones of management.

4.2. PATHOGENESIS OF ALI/ARDS

4.2.1. GENERAL DESCRIPTION OF THE PATHOLOGICAL FEATURES OF ALI/ARDS

Acute lung injury (ALI) and the acute respiratory distress syndrome (ARDS) are syndromes with a spectrum of increasing severity of lung injury defined by physiologic and radiographic criteria in which widespread damage to cells and structures of the alveolar capillary membrane occurs within hours to days of a predisposing insult. This syndrome is characterized by the influx of protein-rich edematous fluid into the airspaces due to increased permeability of the alveolar-capillary barrier. The cells of the alveolar-capillary membrane together with cells of the innate immune and hemostatic systems are targets of damage and effectors of injury in ALI/ARDS [38]. The numbers and morphologic phenotypes of these cells are altered in development of ALI/ARDS and its progression or resolution [114].

The first stage, the exudative phase, is an acute inflammatory response accompanied by a marked influx of neutrophils, and epithelial and endothelial cell injury. The resulting death of epithelial type I cells invites a breakdown in the gas exchange and barrier function of the lung and is associated with the flooding of airspaces with protein-rich edematous fluid with deposition of dense hyaline membranes. Injury to type II epithelial cells reduces surfactant production and impairs the removal of edematous fluid from the alveolar space and favor alveolar collapse. Endothelial injury leads to a widening of cellular junctions and capillary membrane disruption, producing capillary leakage and edema. The proliferative phase is characterized by type II cell proliferation with relining of the denuded basement membrane. This is accompanied by ingrowth of mesenchymal cells, such as fibroblasts, into granulation tissue, followed by the deposition of collagen and the migration of epithelial cells over the surface of organizing granulation tissue. Some patients have an uncomplicated course and the disorder rapidly resolves; however, insufficient repair leads to the fibrotic phase, which is identified by the deposition of excess collagen and extracellular matrices mainly in the alveolar wall [38, 114].

4.2.1.1. Lung epithelial injury

The important role of the alveolar epithelial barrier in the pathogenesis and resolution of ALI/ARDS has been increasingly appreciated, including its role in the production of surface-active material to maintain alveolar stability as well as in the resolution of alveolar edema [107]. The classic ultrastructural studies of Baclofen and Weibel show that epithelial damage was more prominent than endothelial damage in patients who died with ARDS [85, 205]. As the epithelium is exposed to the external environment, it is a target for injury and infection. However, our understanding of the responses of the alveolar epithelium to injury remains incomplete.

Disruption of alveolar epithelial integrity is a major contributor to increased permeability and alveolar flooding with protein-rich edema fluid, a hallmark of ALI/ARDS [82, 107, 110]. The formation and the resolution of protein-rich pulmonary edema is determined by several mechanisms, including lung vascular pressure, lung endothelial and epithelial permeability to protein, and the capacity of the alveolar epithelium to effectively remove alveolar edema fluid and the alveolar proteins. The level of alveolar fluid clearance (AFC) impairment has significant prognosis value in determining morbidity and mortality. Increased alveolar epithelial and endothelial protein permeability, another significant characteristic of ALI/ARDS, can influence the resolution of pulmonary edema. Loss of the epithelial barrier can also lead to septic shock in patients with bacterial pneumonia [206]. The alveolar epithelium not only plays a role in the pathogenesis but also in the resolution of acute respiratory distress syndrome (ARDS) [82, 107, 206]. If the alveolar epithelium damage is severe, disorganized or insufficient epithelial repair may lead to fibrosis [112].

4.2.1.1.1. General features and functions of the alveolar epithelium

The alveolar epithelium not only exerts a barrier function, but also regulates the water and solute content of the fluid lining the epithelium at the air–liquid interface. Regulation of this fluid layer constitutes an essential component of both homeostatic gas exchange and host defense against infection [207, 208]. Under normal conditions, the epithelial barrier is much less permeable than the endothelial barrier [107]. The normal alveolar epithelium is composed of two types of cells. Flat type I epithelial cells make up 90 percent of the alveolar surface area and are easily injured [209]. They serve primarily a barrier function, but are metabolically active, participating in host defense,

alveolar remodeling and antioxidant functions [210, 211]. Cuboidal type II epithelial cells are smaller, make up the remaining 10 % of the alveolar surface area and are more resistant to injury. They are readily recognized by their characteristic lamellar inclusions. These inclusions are the predominant intracellular storage form of surface active material. Physiologic functions of type II cells include: 1) synthesis, storage, and secretion of surface active material; 2) differentiation into alveolar type I cells after injury; 3) hyperplasia and adaptation after lung injury; 4) secretion of non-surfactant proteins; 5) transport of fluid and electrolytes from the alveolar space into the interstitium, and 6) immune functions [1, 156, 205, 212, 213]. The loss of epithelial integrity and injury to type I cells disrupts normal epithelial fluid transport, impairing the removal of edema fluid from the alveolar space [207, 208]. Injury to type II cells reduces the production and turnover of surfactant, contributing to the characteristic surfactant abnormalities [212, 214, 215].

4.2.1.1.2. Alveolar epithelium, protein permeability and alveolar protein clearance in ALI

Increased protein permeability is well documented in ARDS. Alveolar epithelial lining fluid obtained from normal subjects contains a spectrum of soluble proteins derived from plasma [216-220]. Although many of these proteins are quantitatively proportioned as in plasma, very large molecules such as IgM and α2-macroglobulin are much more sparsely distributed to the alveolar surface [219]. In contrast to the normal lung, increased protein permeability in ARDS is associated with loss of this size restriction so that all plasma proteins are distributed equally between plasma and alveolar epithelial lining fluid. The large proteins IgM and α2-macroglobulin were found in ARDS BALF at >90 times the concentrations of normal or cardiac edema fluid, indicating that normal size selectivity is preserved in cardiac edema but is destroyed by the alveolar-capillary injury of ARDS [108]. Patients dying with acute lung injury and the acute respiratory distress syndrome (ARDS) have large quantities of insoluble protein in their air spaces, and patients who die with lung injury after ARDS have three times as much protein in their alveoli as survivors [217, 221, 222]. The alveolar protein clearance is also an important function of the alveolar epithelium to maintain an adequate air-fluid interface. The alveolar epithelium is a tight epithelium and is the primary barrier restricting passage of solutes and water into or out of the alveolar space. The mechanism for the alveolar epithelium to clear molecules as large as serum proteins from the air space is not

totally understood. Given that the rate of transalveolar protein flux is slow and no paracellular passage of large proteins has been observed through the epithelium, it is likely that the proteins are cleared by transcellular pinocytotis [223]. Disruption of the alveolar epithelium is associated with a decrease in the alveolar protein clearance which also impairs the resolution of the lung edema in ALI/ARDS. Clearance of serum proteins from the alveolar space is an important process in recovery from pulmonary edema. Albumin and immunoglobulin G (IgG) are present in pulmonary edema fluid in concentrations that are 40–65% of plasma levels in hydrostatic pulmonary edema and 75–95% in lung injury pulmonary edema. Protein concentrations rise even higher during the recovery phase from alveolar edema because the salt and water fraction of edema fluid is cleared much faster than albumin and IgG [88, 224-228]. High protein concentrations, in turn, increase protein osmotic pressures which slows alveolar fluid clearance. Alveolar liquid clearance slowed from 8%/h in the first 4 h to 3%/h at the end of this period. In addition to limiting liquid clearance, highly concentrated alveolar protein may precipitate, necessitating clearance of insoluble as well as soluble protein. The inability to clear alveolar protein results in the accumulation of fluid and macromolecules in the alveolar space and severe gas exchange impairment. Therefore, it may play a role in poor outcomes after pulmonary edema [225].

4.2.1.1.3. Alveolar epithelium and alveolar fluid clearance in ALI

The classic studies of Matthay and Wiener-Kronish showed that the normal alveolar epithelium can concentrate protein-containing solutions in the airspaces, because electrolytes and water are transported out of the alveolar spaces more rapidly than proteins. Most patients with ALI/ARDS are thought to have altered fluid clearance from the alveolar space. The alveolar fluid clearance is a dynamic balance between fluid formation and clearance across the alveolar epithelium with alveolar liquid volume regulated by a balance between physical and biochemical forces. Edema fluid clearance from the lung occurs when sodium undergoes vectorial transport from the alveoli into the underlying interstitium. This transport is thought to be primarily a function of alveolar type II cells. Transport occurs when sodium enters the cell through the amiloride-sensitive Na channel (ENaC) and is pumped out of the cell by Na/K-ATPases located on the basolateral surface of the epithelial cells [156]. This enzymatic process of sodium transport is manipulable; the Na channel inhibitor amiloride blocks fluid clearance, whereas the ATPase can be stimulated by β-agonists, increasing

sodium transport and therefore trans-epithelial fluid movement [90, 226, 229-232]. Chloride is secreted to preserve electrical neutrality and likely moves through the CFTR present in type I cells [233]. Water follows the osmotic gradient generated by Naþ transport. The specific pathway for water movement across the epithelium is controversial: water may travel through specialized water transporting proteins called aquaporins [234]; however, in transgenic mice where aquaporins have been deleted, alveolar liquid clearance is unaffected [88, 235].

To assess the efficacy of edema fluid clearance from the lung, serial measurements of edema fluid protein concentration must be made. Using this technique, Matthay et al. measured the rate of fluid clearance from the airspaces of the lung in sequential samples of undiluted edema fluid obtained from patients with ARDS [236, 237 92]. A greater capacity to remove alveolar edema fluid early in the course of lung injury was associated with a better outcome. They also completed a larger study of 79 patients with ALI in which they collected pulmonary edema fluid sampled sequentially within the first 4 h after intubation. In this study, they demonstrated by measuring the alveolar fluid clearance that 56% of the 79 patients studied with ALI had impaired alveolar fluid clearance compared to control patients with hydrostatic pulmonary edema [92]. Overall, the mean alveolar fluid clearance rate was 6% per hour. Furthermore, they also showed that in patients with ARDS, the ability to concentrate alveolar fluid proteins in the first hours after the lung injury is a good prognostic sign, whereas patients with unchanging alveolar fluid protein concentrations have a significantly higher mortality [92, 237]. The results confirm that a decrease in the capacity of the alveolar epithelial barrier to remove alveolar edema fluid is an important determinant of a poor outcome [92].

4.2.1.1.4. Other roles of the alveolar epithelium in ALI

One of the most important events for the resolution of ALI/ARDS is the reestablishment of the alveolar epithelium integrity. It is clear that alveolar epithelial type II cells re-populate the injured alveolar barrier in ALI, however, more information is needed about the intracellular signaling pathways that are activated or differential mechanisms of gene expression during injury, proliferation, and differentiation. The cellular denudation of the alveolar epithelium seems to precede and promote activation of fibroblasts which results in the development of fibrosis [87, 238-241]. In addition, current lines of evidence

show that epithelial cells produce cytokines [242, 243], although the mechanisms and the consequent effects are not clear.

4.2.1.2. Lung endothelial injury

The responses of lung endothelial cells are also altered in ALI/ARDS, triggered by sepsis, trauma, and other systemic conditions, but the mechanisms are incompletely defined. The lung endothelium, in concert with the epithelial barrier, mediates the initial change in permeability and is also critical for repair and remodeling of the alveolar capillary membrane. The cells lining the intima of blood vessels comprise an endothelial cell monolayer that functions as a semipermeable barrier, controlling the exchange of macromolecules and fluids between the blood and interstitial space. This tenet is based on experimental data that demonstrate that under basal conditions, the endothelium functions as a barrier to fluid exchange [244], while at the same time it actively translocates diverse macromolecules such as albumin [245], insulin, transferrin [246], ceruloplasmin [247], LDL [248], angiotensin [249], and orosomucoid [250].

Endothelial heterogeneity may be a factor in the lung's responses to pathologic stimuli [251]. In rodent lungs, each short segment of lung capillary possesses a few functionally distinct endothelial cells (the pacemaker cells). These cells appear to regulate endothelial calcium within the capillary segment by generating intercellular calcium waves, and they may be the sites of inflammatory initiation in the capillary [252]. The endothelial cytoskeleton has been identified as a critical regulator of vascular barrier integrity [253]. It has been suggested that the endothelial barrier is regulated by a balance between barrier-disrupting cellular contractile forces and barrier-protective cell–cell and cell–matrix forces [254]. These competing forces exert their opposing effects via manipulation of the actin-based endothelial cytoskeleton and associated endothelial regulatory proteins. Endothelial cells generate tension via actin and myosin fibers, and changes in tension/relaxation of these fibers can be accomplished by regulation of the phosphorylation of myosin light chain (MLC) catalyzed by the Ca2+/calmodulin (CaM)-dependent enzyme myosin light chain kinase (MLCK) [255]. Endothelial proteins, such as thrombin and activated protein C (APC), play an important role on the vascular permeability in lung injury. APC is a vascular endothelial protein which interacts with other coagulation proteins such as thrombin. Whereas thrombin alters the endothelial integrity and consequently increases the endothelial permeability in cultured human pulmonary endothelial cells; the pre- or post-exposure

to physiologic concentrations of APC significantly attenuates the increase in endothelial permeability mediated by thrombin [256]. More needs to be learned about the diversity of endothelial cells, particularly regarding how they regulate the onset of lung inflammation.

It is noteworthy that although the most obvious initial manifestations may be respiratory in nature, ALI/ARDS are part of a systemic process involving microvascular dysfunction of diverse organs including the heart, kidneys, gut, liver, muscle and the brain [184]. Activation of endothelial cells resulting in activation of multiple pathways occurs in both pulmonary and systemic endothelium [257]. We have an incomplete picture of the molecular mechanisms that govern the responses of pulmonary endothelial cells, their interaction with alveolar epithelium, and the responses of the systemic endothelium in ALI/ARDS.

4.2.2. PATHOLOGICAL MECHANISMS

4.2.2.1. Cell death in ALI/ARDS

Cell death has been demonstrated in the lung and other organs during the pathogenesis of ALI/ARDS [96, 258]. The two major pathways of cell death, necrosis and apoptosis, have been described in patients who died with ARDS [85]. These types of cell death have different morphological and biochemical characteristics.

4.2.2.1.1. Description of two modes of cell death: necrosis and apoptosis

Necrosis begins with an impairment of the cell's ability to maintain homeostasis, leading to an influx of water and extracellular ions. Intracellular organelles, most notably the mitochondria, and the entire cell swell and rupture (cell lysis). Due to the ultimate breakdown of the plasma membrane, the cytoplasmic contents, including lysosomal enzymes, are released into the extracellular space leading to an intense inflammatory response that, in turn, can extend the tissue damage. Apoptosis, in contrast, is a controlled form of cell death, in which the cell is an active participant in its own demise ("cellular suicide"). This active process is gene-directed and usually requires protein synthesis. Apoptosis is important in the normal development of tissues

and in the remodeling of tissues that occurs during normal repair processes. Cells undergoing apoptosis show characteristic morphological and biochemical features. The morphological features include cytoskeletal rearrangement, chromatin aggregation, nuclear and cytoplasmic condensation, cell shrinkage, plasma membrane blebbing, and formation of apoptotic bodies (partition of cytoplasm and nucleus into membrane bound-vesicles in which organelles are initially intact). *In vivo*, these apoptotic bodies are rapidly recognized and phagocytized by either macrophages or adjacent epithelial cells, so intracellular products are not spilled into the extracellular space. Biochemically, activation of specific intracellular serine rich proteases (caspases) leads to DNA cleavage, which is quite different from the random DNA degradation observed in necrosis [241, 258-261].

Necrosis is a degenerative phenomenon that follows irreversible injury. Necrosis occurs when cells are exposed to extreme conditions such as hyperthermia, trauma or severe hypoxia, which may result in damage to the plasma membrane [259, 262]. In the lung, necrosis caused by hypoxia is less common because of the dual pulmonary circulations and the ventilated alveolar spaces [241]. Other agents like complement, lytic viruses and some bacterial exotoxins (toxins released by *Pseudomonas* species, *Escherichia coli* or *Staphylococcus aureus*) can cause direct lysis of the epithelial cells in the lung [263, 264]. Mechanical forces created by shear stress or overdistension may also cause direct disruption of epithelial membranes, particularly in injured lungs in which heterogeneous alveolar flooding leads to marked heterogeneity in regional compliance [264, 265]. Apoptosis signaling pathways are activated in response to receptor-ligand interactions (e.g., tumor necrosis factor-receptor family), environmental factors (such as ultraviolet light and changes in oxidation/reduction potential), and internal factors encoded in the genome ("programmed cell death"). Inappropriate activation or inhibition of these pathways can lead to disease, either because cells have prolonged survival (malignant transformation) or because they die prematurely and produce structural changes in the tissue (e.g., apoptosis of lung epithelial cells) [241, 258].

4.2.2.1.2. Apoptosis of alveolar epithelial cells in ALI

The lung is a complex organ which includes many different types of cells; including endothelial cells, epithelial cells, fibroblasts, and inflammatory cells [266-268]. Apoptosis can either ameliorate or exacerbate lung injury, depending upon the cell type.

Excessive apoptosis might contribute to injury of both the alveolar epithelial and endothelial barriers. In patients with sepsis, apoptosis has been postulated to be a major mechanism of end-organ damage and has been demonstrated in the intestinal epithelium and the spleen of patients dying from sepsis [269-271]. By contrast, inadequate apoptosis might impair the resolution of type II pneumocyte hyperplasia or the removal of activated inflammatory cells, leading to an inadequate repair and prolonged inflammation [241, 258]. The alveolar epithelium of patients who die from lung injury contains cells that exhibit morphological and biochemical features of apoptosis such as DNA fragmentation [272], and upregulation of the intracellular pro-apoptotic pathway (*Bax*, a *Bcl-2)* [273]. Evidence of extensive alveolar epithelial cell apoptosis has been described in murine models of pulmonary fibrosis and lipopolysaccharide-induced lung injury [274-277]. Apoptosis of alveolar epithelial cells is detectable in mice as early as 6 hours after intratracheal administration of lipopolysaccharide [277]. Apoptotic pathways are modulated by cytokines and other inflammatory products. At inflammatory sites in the lungs, neutrophil apoptosis is delayed and epithelial apoptosis is enhanced [95, 96, 278, 279]. Because apoptosis is a regulated event, new therapeutic interventions that inhibit key steps in the apoptosis pathways hold promise for limiting some types of injury responses in the lungs [267, 280].

Apoptosis can be initiated by activation of a family of death receptors (extrinsic pathway) or by direct mitochondrial damage (intrinsic pathway). Death receptors include Fas (CD95) and tumor necrosis factor receptors (TNF-I and -II) on the cell surface, which are triggered by protein ligands either on the surface of effector cells, or in the soluble phase of extracellular fluids. Activation of these membrane receptor proteins mediates apoptosis via activation of intracellular caspases, resulting in the cleavage of nuclear DNA. The cognate Fas Ligand (FasL) is a membrane protein that can be shed from cell membranes by metalloproteinases (MMP-7 and MMP-3) and accumulates in extracellular fluids as a soluble ligand (sFasL). Studies of human lung tissue have shown that Fas is present on proliferating type II pneumocytes, as well as airway epithelial cells [280-283]. Activation of membrane Fas caused apoptosis of murine type II cells *in vivo*. The bronchoalveolar lavage fluid was found to contain sFasL before and after the onset of ARDS, but the sFasL was biologically active only at the onset of ARDS [284]. Other receptor-mediated apoptosis pathways also exit. In this line, Uhal et al. have found that angiotensin peptides (angiotensin I to angiotensin II)

produced by fibroblasts from fibrotic human lungs initiated apoptosis of alveolar epithelial cells via the angiotensin II receptor [268, 285]. In ARDS the concentration of angiotensin converting enzyme, which catalyzes the conversion of angiotensin I to angiotensin II, is increased in BAL fluid [286]. On the other hand, surfactant protein A (SP-A), the primary protein present in pulmonary surfactant, is an inhibitor of type II apoptosis *in vivo* [287, 288]. This is important because, in patients with early ARDS, the concentration of SP-A is decreased in BAL fluid [214]. The lower concentration of SP-A would favor apoptosis of type II cells in these patients. Therefore, in early ARDS a combination of three factors favors alveolar epithelial apoptosis: increased concentrations of soluble Fas ligand; increased concentrations of angiotensin-converting enzyme and angiotensin II; and decreased concentrations of SP-A [289].

Apoptosis can also be initiated by direct mitochondrial damage [290]. Either hyperoxia or hypoxia can induce apoptosis of pulmonary epithelial cells in experimental systems, and both are relevant in patients with ALI who are ventilated with a high FiO_2, yet at the same time may have regions of tissue hypoxia where alveolar collapse has occurred. Hyperoxia induced apoptosis of primary rat alveolar epithelial cells *in vitro* by activating the pro-apoptotic protein (Bax) at the mitochondrial membrane [291]. This leads to the release of mitochondrial cytochrome C, which binds to Apaf-1 and activates caspase 9, and subsequently caspase 3, followed by DNA fragmentation and apoptotic cell death. In contrast, exposure of rat type II cells to graded hypoxia suppresses proliferation and causes apoptosis by a mechanism that involves activation of hypoxia inducible factor (HIF-1α), and the hypoxia response element (HRE) in nuclear DNA [241, 292].

One of the consequences of apoptosis is loss of cellular attachment to the underlying basement membrane. This could result in the exposure of the underlying alveolar epithelial basement membrane to inflammatory products in the alveolar spaces, such as oxidants, proteinases, and inflammatory factors. Destruction of the alveolar walls and activation of fibroblast proliferation and collagen production, which is known to occur at the onset of ALI in humans, could lead to fibrosis during the repair process [83]. Current studies are attempting to identify the mechanisms that link apoptosis with acute and chronic fibrosis in the lungs [238, 241, 293-298].

4.2.2.1.3. Apoptosis of PMN in ALI

Polymorphonuclear leukocytes (PMN) undergo apoptosis, and macrophages can remove apoptotic PMN by phagocytosis [96, 299-304]. However, inflammation appears to blunt apoptosis of PMN cells *in vitro* and *in vivo* studies [304-310]. Despite the large content of PMN infiltrating the lung tissue in acute lung injury, G. Matute-Bello and T.R. Martin (mentor group) found that there are fewer apoptotic PMN in bronchoalveolar lavage (BAL) fluid of patients either with acute respiratory distress syndrome (ARDS) or at risk of ARDS [96, 282, 284, 289]. Furthermore, BAL from ARDS patients decreases apoptosis and prolongs survival *in vitro* of normal human PMN, due to the presence of anti-apoptotic soluble factors, such as the proinflammatory cytokines granulocyte colony-stimulating factor and granulocyte/macrophage colony-stimulating factor [95], and possibly IL-8 and IL-2 [96, 311-313]. These observations suggest that reduction of PMN apoptosis may prolong inflammatory responses and predispose the patients to ARDS after acute lung injury [314-316]. Interestingly, apoptotic PMN are taken up rapidly by activated alveolar lung macrophages, which indicates that two processes are likely to be occurring simultaneously: PMN apoptosis is delayed in the airspaces, and apoptotic PMN are taken up very rapidly by activated macrophages [96, 258, 278, 289].

4.2.2.2. Inflammation

Acute lung injury (ALI) and its more severe form, the acute respiratory distress syndrome (ARDS), reflect severe injury leading to dysfunction of the barrier properties of the pulmonary endothelium and epithelium as a consequence of an unregulated acute inflammatory response [85, 317]. ALI/ARDS is characterized by an acute inflammatory process in the airspaces and lung parenchyma. With initiation of inflammation there is increased leukocyte production and rapid recruitment to the inflamed site [82]. There is also activation of mediator cascades including the production of cytokines, chemokines, acute phase proteins, free radicals, complement, coagulation pathway components, and focal upregulation of adhesion molecule expression. During the early phase of ALI, a variety of inflammatory mediators are released into the distal air spaces that can initiate and amplify the inflammatory response [38, 129, 317-321]. For example, cytokines such TNF-α, IL-1, and IL-8, are produced locally in the lung by alveolar macrophages, epithelial cells, and fibroblasts, and have been involved in epithelial and endothelial cell injury [82, 322-324]. Other

proinflammatory mediators, including endotoxin (LPS), vascular endothelial growth factor (VEGF), high mobility group-1 protein, and thrombin, are also implicated in the pathogenesis of ALI/ARDS. To regulate the pro-inflammatory response, there is also activation of "antiinflammatory" responses including glucocorticoids, cytokines (interleukin (IL)-4, IL-10 and IL-1 receptor antagonist (IL-1RA) and other mechanisms, such as shedding of adhesion molecules. These antiinflammatory mediators limit the proinflammatory response in the injured lung [82, 325 326 319, 321, 327, 328]. The balance between pro- and anti-inflammatory factors will determine the net effect of inflammation in the lung.

4.2.2.2.1. Neutrophils

Neutrophils are the dominant leukocytes found both in BAL fluid and in histological specimens from patients with ARDS [82, 96, 282, 284, 289, 329]. Some lines of evidence consider that an initiating event (sepsis, shock, trauma, multiple transfusions, pancreatitis, etc.) leads to activation of pulmonary epithelium, endothelium and macrophages (alveolar and interstitial) resulting in upregulation of adhesion molecules and production of cytokines and chemokines that induce a massive sequestration of neutrophils within the pulmonary microvasculature. These cells transmigrate across the endothelium and epithelium into the alveolar space and release a variety of cytotoxic and proinflammatory compounds, including proteolytic enzymes, reactive oxygen species (ROS) and nitrogen species, cationic proteins, lipid mediators, and additional inflammatory cytokines [279, 319, 325, 326, 330-332]. This perpetuates a vicious cycle by recruiting additional inflammatory cells that in turn produce more cytotoxic mediators, ultimately leading to profound injury to the alveolo-capillary membrane and respiratory failure.

In many animal models the degree of lung injury is reduced dramatically if neutrophil influx is abated, although this is not a universal finding [333, 334]. However, ARDS can develop in neutropenic patients [335], and neutrophils can enter the lungs of nomal subjects without altering epithelial permeability [336]. Because some animal models of acute lung injury are neutrophil-independent, neutrophils are believed to be an important but not essential component of the injurious response [337]. Neutrophils cause cell damage through the production of free radicals, inflammatory mediators, and proteases. Excessive quantities of neutrophil products including elastase, collagenase,

reactive oxygen species, and cytokines, such as tumor necrosis factor-α (TNF-α) have been found in patients with ARDS [279, 319, 325, 326, 330-332, 338]. Recent studies with mice deficient in neutrophil elastase suggest that this enzyme may be an important mediator of damage to the alveolar epithelium and the progression to fibrosis [339]. Adhesion molecules, notably β_2-integrins, mediate neutrophil binding to the pulmonary endothelium. Adhesion molecules also modulate activation and mediator release by neutrophils. Other inflammatory cells including macrophages and, later, lymphocytes are involved, while platelets may exacerbate the vascular injury and endothelial cells themselves are capable of producing many damaging mediators of inflammation [340].

4.2.2.2.2. Inflammatory mediators

a) Cytokines

Evidence from several clinical studies indicates that a complex network of inflammatory cytokines and chemokines (chemotactic cytokines) play a major role in mediating, amplifying, and perpetuating the lung injury process. Cytokines are low-molecular-weight soluble proteins (generally < 30 kDa) that transmit signals between cells [319]. Proinflammatory cytokines are produced locally in the lung inflammatory cells, lung epithelial cells or fibroblasts. Cytokines production by extrapulmonary factors has also been described. Simultaneous production of anti-inflammatory cytokines can potentially counteract pro-inflammatory cytokine effects and modify the intensity of the inflammatory process. The cytokine balance between proinflammatory and anti-inflammatory cytokines is crucial to understand the biological activity of cytokines in biological fluids [319, 327].

The inflammatory process is driven in part by cytokines including TNF-α, IL-1β, IL-6, and IL-8. All have been found in BAL fluid and plasma of patients with ARDS [322, 341, 342]. TNF-α and IL-1β seems to be the earliest released cytokines in the lungs with acute lung injury. The increase in TNF-α levels occurs very early in the clinical course of induced acute lung injury and may be missed by the time of presentation. TNF-α and IL-1β are present in BAL fluid of patients at risk for ARDS and with established ARDS [341, 343, 344]. The highest concentrations of TNF-α and IL-1-β occur in the BAL fluid from patients with sustained ARDS. The ratios of BAL fluid to serum cytokine concentrations are typically elevated, suggesting a pulmonary origin [345, 346]. In animal models, both cytokines can produce an ARDS-like condition. They are produced by

inflammatory cells and can promote neutrophil-endothelial adhesion, microvascular leakage, and amplify other proinflammatory responses.

The pro-inflammatory cytokines IL-1β and TNF-α identified in BAL fluids from ARDS patients are also accompanied by high concentrations of their specific antagonists IL-1 receptor Antagonist (IL-RA) and soluble TNF receptors (sTNFR) [129]. This prominent antiinflammatory response in the lungs provides a mechanism for limiting the intensity of the inflammatory response in the soluble phase of lung fluids before and after the onset of ARDS. The balance of "anti-inflammatory" cytokines and mediators must also be considered. Some studies using a bioassay that measures balance of cytokines with their inhibitors, determined that the proinflammatory activity in the BAL fluids from patients with ARDS is mainly due to IL-1β, rather than to TNF-α that is considerably less active than IL-1β [317, 323, 341]. Therefore, IL-1β seems to be one of the most biologically active cytokines in the lungs early after the onset of ALI. In experimental models, IL-1β stimulates the production of a variety of chemokines (e.g. IL-8, monocyte chemotactic protein-1 (MCP-1) and macrophage inflammatory protein-1α (MIP-1-α))[321] involved in epithelial wound repair [323, 347] and is a potent inducer of lung fibrosis [348, 349]. It has been previously shown in rats that lung vascular permeability increases after short term exposure of IL-1α and IL-1β protein when given intratracheally [350, 351].

b) Chemokines

The intense neutrophilic infiltrate has led to a search for the chemotactic factors responsible. Leukocyte migration is directed to a large extent by chemokines (chemotactic cytokines). The two major classes include the α-chemokines, which recruit PMNs, and β-chemokines, which recruit monocytes and lymphocytes [342]. The α-chemokines include IL-8, GRO (melanoma growth stimulating activity), and ENA-78 (epithelial cell neutrophil activating factor). The β-chemokines include the monocyte chemotactic peptides (MCP-1,2,3,4) and RANTES (regulated on activation, T-cell expressed and secreted). IL-8, GRO, and ENA-78 are detectable in the BAL fluid of patients at risk for ARDS and during the course of established ARDS. Alveolar macrophages are a major source of chemokines in the airspaces, and produce IL-8, GRO-related peptides, and ENA-78. The α- and β-chemokines are present in the lungs of patients with ARDS. IL-8, GRO, ENA-78, and MCP-1 all have been found in BAL

fluid of patients at-risk for and with established ARDS [346, 352]. Although other potent leukocyte chemoattractants also exist, including the complement component C5a and the low-molecular-weight lipids leukotriene B_4 and platelet activating factor, the neutrophil chemotactic activity in BAL fluid is due predominantly to IL-8, and not to C5a [346, 353]. Correlations between IL-8 and total PMN in BAL fluid is poor at the onset of ARDS, however, it actually grows stronger with time in patients with persistent ARDS [346, 353]. Other chemokines in ARDS BAL fluid also are likely to contribute to PMN recruitment such as GRO and ENA-78. Despite the fact that the concentrations of GRO and ENA-78 exceed the concentration of IL-8 in BAL fluid throughout most of the course of ARDS [346, 352], IL-8 was the dominant PMN chemoattractant in the fluids studied. GRO and ENA-78 may then account for IL-8 independent neutrophil adhesion in ARDS. MCP-1, which regulates monocyte recruitment, is detectable in ARDS BAL fluid at the onset of ARDS and persists in the lungs of patients with sustained ARDS [346]. These neutrophil chemotactic cytokines are also balanced by the presence of counter-ligands such as α_2-macroglobulin, the Duffy antigen chemokine binding protein, natural neutralizing antibodies, and the anti-inflammatory cytokine IL-10 in the lung milieu. The macrophage migration inhibitory factor (MIF) was identified in BAL fluid from patients studied on the first day of ARDS [354], and increases in the lungs of patients with sustained ARDS [321]. Activated alveolar macrophages of patients with ARDS seem to produce this cytokine. MIF may sustain inflammation in the alveolar spaces, although the role of MIF in the lungs of patients with ARDS is unclear.

Molar concentration ratios of pro- and anti-inflammatory molecular pairs in BAL fluid provide insight into the inflammatory balance in individual patients, and measurement of the net inflammatory activity in the lungs of patients with ARDS is an important step in the pathogenesis of this syndrome. Low concentrations of IL-10 and IL-1RA in BAL fluid from patients with ARDS were found to be associated with increased mortality, suggesting an important role for anti-inflammatory mediators in counter balancing the pro-inflammatory response [355]. Therefore, it is possible that ARDS represents the failure of anti-inflammatory cytokine responses in susceptible individuals as much as the activation of pro-inflammatory networks.

c) Other mediators

The toll-like receptors of innate immune cells and endothelial cells, which recognize pathogen-associated molecular patterns in LPS and other microbial factors, are

another group of factors that are critical to inflammation and injury. Toll-like receptors are linked by intracellular signal transduction cascades to activation of the nuclear factor-κB (NF-κB) family of transcription factors and induction of tumor necrosis factor alpha and other NF-κB dependent gene products. Their engagement is central to host defense and sepsis, septic shock, and their complications including ALI/ARDS [356].

Bacterial lipopolysaccharide (LPS) is a component of the gram-negative cell wall that activates innate immunity [357]. Most experimental models of ARDS have used LPS to trigger inflammatory responses by interacting with specific host proteins that initiate cytokine production. During the acute phase of ALI, concentrations of the lipopolysaccharide-binding protein (LBP) and sCD14 in plasma and bronchoalveolar lavage fluid rise by more than ten-fold in patients with acute lung injury [358]. LBP enhances several LPS-stimulated responses that could contribute to ARDS, including the regulation of CD18, the adherence of neutrophils to endothelium, and the production of cytokines (e.g., TNF-α from alveolar macrophages). The LPYLBP complex interacts with CD14 to trigger intracellular responses and the development of lung injury. The increased levels of LBP and CD14 in BAL fluid from patients with ARDS, are significantly related to total protein and polymorphonuclear neutrophil concentrations, markers of increased alveolar-capillary membrane permeability and inflammation, respectively [359]. LPS can also induce tolerance to inflammatory insults. Exposure to a sublethal dose of LPS is associated with relative hyporesponsiveness to subsequent exposure. The extent to which endotoxin tolerance could modulate the development of ARDS in individuals at risk remains unclear but represents an exciting therapeutic prospect. Finally, although LPS is implicated as an important agent in the induction of ARDS and is present in the plasma of many patients with sepsis complicated by lung injury, its precise source remains unknown [357].

4.2.2.3. Metalloproteinases

The response to acute lung injury is characterized by a series of events, including inflammation and extracellular matrix (ECM) revision, which can result in loss of the normal alveolar architecture. Throughout this process, ECM components are deposited, removed, or remodeled, enabling cell migration, neovascularization, and restructuring of the alveolar-capillary interface. These MMPs are thought to make important contributions to the inflammatory response [360-365]. These proteolytic enzymes

have important functions in normal developmental processes, such as lung branching morphogenesis. On the other hand, patients with acute respiratory distress syndrome (ARDS), asthma or fibrosis lung diseases express increased levels of MMPs, suggesting their participation in the response to lung injury [30, 366]. In pathological conditions, MMPs are involved in inflammatory processes, tissue injury and repair, remodeling, and host defense against pathogens [367-369]. Some of the known targets of MMPs in the lung include extracellular matrix (ECM) molecules, growth factors, chemokines, proteinases, and cell surface proteins, such as adhesion molecules and TNF family ligands. The proteolytic activities of MMPs must be tightly controlled to restrict inflammation and ECM revision to areas of lung damage and to avoid destruction of healthy tissue. An important mechanism of MMP regulation is provided by the tissue inhibitors of metalloproteinases (TIMPs), which block MMP proteolytic activity. Four homologous TIMPs have been well characterized: TIMP-1,-2, -3, and -4 [370, 371]. The functional significance of the interactions between MMPs and their substrates in bronchoalveolar fluid and in the lung parenchyma is a novel concept that is currently being explored, but remains poorly understood.

The MMP-2, MMP-7, MMP-9, and tissue inhibitor of metalloproteinase (TIMP)-1 have been demonstrated in pulmonary epithelium in patients with asthma, ARDS, and cystic fibrosis [372-374]. In patients with ARDS, MMP-9 (92 kDa-gelatinase B) and MMP-2 (72 kDa-gelatinase A) are also elevated in the BAL fluid, and the number of neutrophils present in the BAL fluid correlates with the increase in MMP-9 [375, 376]. In addition, the elevated levels of MMP-9 and MMP-2 in the BAL fluid of these patients correlate directly with an increase in the concentration of degradation products from type IV collagen within the basement membrane. Fligiel et al. detected high levels of MMP-2, MMP-8 (neutrophil collagenase), MMP-9, and TIMP-1 (metalloproteinase innhibitor-1) in all the BAL fluid of all patients with ALI/ARDS included in the study, whereas MMP-1 (interstitial collagenase) and/or MMP-3 (stromelysin-1) were detected only in a small subset of patients. In these patients with elevated levels of MMP-3 and/or MMP-1, the severity of illness and overall mortality rate were higher than in the group in which only MMP-2, MMP-8, and MMP-9 were detected [370].

In the lung, MMPs are produced by a wide variety of cell types, including type II epithelial cells, airway epithelial cells, endothelium, fibroblasts, and alveolar macrophages. MMPs derived by migrating neutrophils could also play pathogenic

roles in acute lung disease. MMP-8, generated by neutrophils, has been identified in BAL fluid of adult and neonatal patients with ARDS. In these studies, elevated MMP-8 was associated with the presence of large numbers of neutrophils in lung tissue [377-379]. Gibbs et al., however, determined that alveolar macrophages isolated from injured lungs produced the same spectrum of MMPs as seen in the BAL fluid from patients with ARDS. These studies suggest that alveolar macrophages can be the main source of MMPs in the lung under pathological conditions, and that MMPs can play an active role in the development of lung injury [380, 381].

In animal models of inflammatory lung diseases induced by IgG-containing immune complexes [382] or LPS [381], administration of TIMP-2 via intratracheal instillation resulted in a reduction of lung injury associated with a significant reduction of both the neutrophil influx to the alveolar space and the protein permeability index of the lung. One mechanism involves a direct role for MMPs in neutrophil recruitment. The presence of exogenous MMP inhibitor in the alveolar space may limit neutrophil influx in a variety of possible ways. The presence of the inhibitor may prevent damage to the basement membrane, which is necessary for neutrophil transmigration across the vessel wall. Such an effect might also account for the reduction in protein permeability. Another possibility is that the proteolytic enzymes might be directly responsible for injury to the resident epithelial cells and endothelial cells. Injury to resident cells in the alveolar unit is a pathogenic feature of acute inflammatory lung injury. Cellular injury has been directly attributed to proteolytic enzymes alone or the combinations of oxidants and proteolytic enzymes [380, 381, 383]. In a mouse model of Bleomycin-induced lung injury, TIMP-1 deficiency amplifies acute lung injury with increased pulmonary neutrophilia, hemorrhage, and vascular permeability. These experimental models of lung injury suggest an important role of MMPs in the development of acute lung injury by promoting leukocyte influx and alteration of the vascular permeability at sites of inflammation [381, 382, 384, 385].

Within the MMP family, however, matrilysin (MMP-7) is the only known member that lacks the hemopexin domain thought to be important in interactions with TIMPs, whereby it is believed to be less sensitive to the inhibitory action of these proteins [386]. In addition to its activity on basement membrane and extracellular matrix components, matrilysin (MMP-7) has been reported to cleave a variety of secreted and cell-surface proteins [387]. These cell-surface substrates include the transmembrane precursor to

TNF-α, and FasL. Cleavage of FasL by matrilysin results in the release of active soluble Fas ligand (sFasL) from the cell surface [388]. Matrilysin (MMP-7) is mainly expressed in the cell-surface of epithelial cells. MMP-7, unlike many MMPs, is expressed by non-injured, non-inflamed mucosal epithelia in most adult human tissues [389, 390]. In the human lung, matrilysin is constitutively expressed in tracheal glands and in tracheobronchial epithelium, and expression is acutely up-regulated by injury or exposure to bacteria [391, 392]. Matrilysin is induced in alveolar epithelium in mice after lung injury with bleomycin, and expression increases and continues as fibrosis progresses [384]. This MMP has been reported to contribute to the development of fibrosis in the lung [393, 394]. Despite this harmful effect, matrilysin regulates neutrophil influx by controlling chemokine compartmentalization during the first days after injury, and mediates shedding of E-cadherin that is required for lung epithelium repair [395]. Therefore, it is not clear whether MMP inhibition is beneficial or harmful and in which situations it would be of clinical help.

4.2.2.4. Coagulation

Disruptions in the coagulation system have long been recognized to be an integral part of inflammation, sepsis and ALI. Elemental to the pathophysiology of sepsis and ALI is a shift towards a pro-coagulant state. Bronchoalveolar (BAL) fluid from patients with ALI reflects this increase in procoagulant activity with elevated levels of fibrinopeptide A, factor VII and d-dimer. Concomitantly, there is a decrease in fibrinolytic activity, as shown by depressed BAL levels of urokinase and increased levels of the fibrinolysis inhibitors plasminogen activator inhibitor (PAI) and α2-antiplasmin [396, 397]. This pro-coagulant milieu contributes to the pathophysiology of ALI by causing intra-alveolar and interstitial fibrin deposition which can, in turn, lead to direct endothelial contraction [398]. Moreover, fibrin inhibits surfactant, which contributes to alveolar collapse, and promotes the aggregation of fibroblasts leading to increased fibrosis. In addition, fibrin is also a chemotactic factor for neutrophils [399, 400]. Indeed, fibrin microthrombi are present at autopsy in patients with acute respiratory distress syndrome (ARDS) [49].

Over the past decade, the protein C pathway has been increasingly recognized as an essential participant in both coagulation and inflammation, stimulating research into its role in sepsis and ALI. In the landmark Prowess study, a recent multi-center clinical trial, activated protein C (APC) treatment resulted in a significant survival benefit in

patients with sepsis [168]. APC inactivates the active forms of factor Va and VIIIa which, in turn, blocks downstream thrombin generation. The protein C pathway also has important anti-inflammatory properties; APC blocks nuclear factor-κB nuclear translocation and decreases TNF-α production in mice exposed to endotoxin. Clinically, patients with sepsis and low protein C concentrations are more likely to progress to shock and have a higher mortality than patients with higher levels [401, 402]. However, in humans given endotoxin, APC infusion results in improved hemodynamics but has no effect on inflammatory, thrombotic or fibrinolytic markers [403, 404], suggesting an alternative protective mechanism. The mechanism by which APC has a protective effect is not completely understood.

4.2.2.5. Oxidative stress in ALI

In ALI/ARDS, neutrophil migrates into the alveolar spaces and releases a variety of cytotoxic and proinflammatory compounds, including reactive oxygen species (ROS) and nitrogen species (RNS) in addition to cytokines, proteolytic enzymes and lipid mediators [330, 405]. Both ROS and RNS help perpetuate the inflammatory response, and contribute to alveolo-capillary membrane damage and respiratory failure.

Biologically important, ROS include superoxide anion radical (O2-), hydrogen peroxide (H2O2), hydroxyl radical (OH -), and hypochlorous acid (HOCl). Reactive nitrogen species, derivatives of nitric oxide (NO) including peroxynitrate (ONOO -), have also been implicated in oxidation/nitration of proteins and lipids [406-411]. Reactive oxygen and nitrogen species can lead to cell injury by various mechanisms, including: 1) direct damage to DNA resulting in strand breaks and point mutations; 2) lipid peroxidation with formation of vasoactive and pro-inflammatory molecules such as thromboxane; 3) oxidation of proteins (primarily at sulfhydryl groups) that alter protein activity [412-414], leading to release of proteases and inactivation of antioxidant and antiprotease enzymes [415]; and 4) alteration of transcription factors such as activator protein-1 and nuclear factor (NF)-κB, leading to enhanced expression of proinflammatory genes [416].

To neutralize free radicals and counteract the detrimental effects of ROS generated during normal metabolism, cells express a number of endogenous antioxidants such as superoxide dismutase (SOD, which degrades O_2-), catalase, and glutathione peroxidase (GSH, which inactivates H_2O_2 and hydroperoxides, and is particularly

abundant in the lung) [405]. However, these antioxidants are rapidly overwhelmed during an acute inflammatory response. In the context of ALI/ARDS, there are many potential sources of ROS, including itinerant and resident leukocytes (neutrophils, monocytes, and macrophages), parenchymal cells (endothelial and epithelial cells, fibroblasts, and myocytes), circulating oxidant-generating enzymes (xanthine oxidase), and inhaled gases with high concentrations of oxygen that are often used during mechanical ventilation. Patients with ARDS have increased levels of H_2O_2 in exhaled breath condensate [417-419]. Moreover, bronchoalveolar lavage fluid from these patients contains an excess of oxidatively modified proteins in combination with a relative deficiency in antioxidant molecules such as glutathione [420-423]. Thus, although there is a complex antioxidant defense system, in ALI/ARDS there is extensive overproduction of ROS to the extent that endogenous antioxidants are overwhelmed, permitting oxidative cell damage. Experimental models also support the role of oxidants and oxidative injury in the pathogenesis of ALI/ ARDS [159, 332, 409, 417, 424].

4.2.2.5.1. Source of oxidants in the lung

Activated neutrophils and macrophages, in the bronchoalveolar space are generally considered to be the most potential sources of ROS [425] such as superoxide anion, hydrogen peroxidase, hydroxyl radicals, and hypochlorous acid (HOCl), which are highly toxic to extracellular microorganisms and tissues [332]. The BAL fluids of ARDS patients and at-risk patients contain a variety of pro-inflammatory compounds, including lipopolysaccharide (LPS), cytokines, chemokines, complement fragments, clotting fragments, and lipid mediators, which are capable of priming and/or activating neutrophils to generate ROS. Leukocytes express three enzyme systems, the NADPH oxidase [426], nitric oxide synthases (NOS) [427] and myeloperoxidase (MPO), which can generate reactive species in substantial amounts [428, 429]. The large numbers of activated neutrophils in the lung in ALI/ARDS has focused attention on these phagocytes as a major source of ROS [428]. In addition, it has been described that fibroblasts, endothelial, epithelial, and smooth muscle cells have also phagocytic functions and express oxidases capable of generating physiologically important amounts of ROS [419, 430-432]. Other sources of ROS include mitochondrial electron transport chain [433], cytochrome P450 [434], and xanthine oxidase [435]. Finally, inhaled oxidants, including high concentrations of oxygen, used in mechanical ventilation can contribute to formation of ROS [332].

4.2.2.5.2. Potential targets of oxidants in ALI/ARDS

Numerous changes that occur during the evolution of ALI are the consequences of oxidative stress that leads to cellular damage. The delicate balance between oxidant and anti-oxidant systems is very important in maintaining the normal function and structure of the lung.

a) Oxidation of cellular components

Various cellular components can be subject to oxidative modifications including membrane, cytosolic, and nuclear lipids and proteins. Also nucleic acid can be modified by oxidation. Cellular membranes and especially plasma membranes are primary targets of ROS. The fatty acid side chains of membrane phospholipids undergo peroxidation under oxidative stress [436]. Membrane fluidity is dependent on lipid composition of the plasma membrane, and alterations in this composition, including those by oxidation, profoundly influence diverse aspects of membrane function. In the context of acute inflammation, oxidation of components of the endothelial or epithelial plasma membrane could facilitate neutrophil recruitment into the lung by compromising the barrier function of these cells, thereby allowing leakage of chemokines and other chemoattractant molecules into the vascular space. Additionally, oxidant exposure can lead to enhanced leukocyte adhesion either by direct oxidative modification of components of the endothelial plasma membrane generating lipid mediators or by "inside-out" signaling leading to enhanced surface expression and affinity of adhesion molecules [437]. Cytosolic and nuclear events initiated by oxidants might also contribute to inflammatory injury and be amenable to pharmacologic intervention. For example, elevation of cytokines and chemokines is a common feature in the lung of patients with ALI/ARDS, and it is primarily regulated by NF-κB, a DNA-binding factor that stimulates transcription of many different cytokines involved in acute inflammation. There is evidence that NF-κB is activated in the context of ALI/ ARDS. Oxidation of IκB, the inhibitor factor of NF-κB, results in dissociation of IκB from NF-κB, which allows the free NF-κB to translocate to the nucleus [438-440]. Attempts to attenuate lung injury have focused on modulation of the signaling pathways leading to increased inflammatory cytokine/chemokine production, and on restoration of the oxidant/antioxidant balance to limit the degree of oxidative cell damage [332, 441].

b) Oxidation of extracellular proteins

Accumulation of oxidized proteins occurs in many diseases such as Alzheimer's disease, arteriosclerosis, amyotrophic lateral sclerosis, muscular dystrophy or rheumatoid arthritis. In the lung, the increased production of ROS leads to an oxidative modification of proteins in the alveolar space in patients with ALI [423, 442, 443]. A.G. Lenz et al. determined that oxidatively modified proteins clearly accumulate in BAL fluid of ARDS patients, and to a minor extent in at-risk patients [423]. Oxidative modification of proteins is an early event in oxidative damage to endothelial cells, which suggests that oxidative modification of proteins is not only a marker for oxidative stress but a causal factor in oxidative injury [443, 444]. In this sense, the presence of oxidized pulmonary proteins may be important in the pathogenesis of ARDS. Carbonyl formation in the amino acid side-chains of proteins is one of the consequences of oxidation and appears to be the first change occurring with low amounts of oxidant. Increase in carbonyl groups has been detected in the proteins accumulated in the BAL fluid of patients with ARDS. The formation of these carbonyl groups has been used as a marker of oxidative stress damage in multiple tissues included the lungs. However, carbonyl groups cause loss of function in the affected proteins and also lead to protein degradation contributing to protein-turn-over [423, 444, 445]. In patients with ARDS, there is a correlation of oxidized proteins with the number of neutrophils and myeloperoxidase suggesting an important role of neutrophils in this oxidation process. In fact, carbonyls are generated on proteins by myeloperoxidase-derived hypochlorous acid, a reaction that takes place mainly in neutrophils [408, 423, 429, 446, 447].

4.2.2.6. Fibrosing alveolitis

After the acute phase of acute lung injury and the acute respiratory distress syndrome, some patients have an uncomplicated course and rapid resolution of the disorder [237]. Others have progression to fibrotic lung injury, and such injury can be observed histologically as early as five to seven days after the onset of the disorder [85, 109, 110]. The alveolar space becomes filled with mesenchymal cells and their products, along with new blood vessels [400]. The finding of fibrosing alveolitis on histologic analysis correlates with an increased risk of death, [448] and patients who die of the condition have a marked accumulation of collagen and fibronectin in the lung at autopsy [113].

Recent evidence suggests that there is a much greater overlap of the inflammatory and fibroproliferative phases than previously thought [5], and many mediators are common to both processes. The process of fibrosing alveolitis apparently begins early in the course of the disorder and may be promoted by early proinflammatory mediators such as interleukin-1 [449-452]. Levels of procollagen III peptide, a precursor of collagen synthesis, are elevated in the alveolar compartment very early in the course of the illness [83, 84]. High concentrations of PCPIII in BAL are associated with an increased risk of death, suggesting more severe lung injury [84]. Transforming growth factor-alpha (TGF-α) increases fibroblast collagen production and may contribute to the fibrosis that occurs in patients with sustained ARDS [98]. TGF-α is detectable in BAL for prolonged periods after the onset of ARDS, and high concentrations of TGF-α in BAL on day 7 are associated with an increased risk of death in patients with sustained ARDS [453]. TGF-α also stimulates epithelial cell proliferation *in vitro* [454]. Other growth factors that stimulate epithelial cell proliferation, including keratinocyte growth factor and hepatocyte growth factor, have been detected in ARDS BAL [455]. Myofibroblast cells also show an early increase in the alveolar walls, and BAL fluid from ARDS patients within 48 hours of diagnosis is intensely mitogenic for fibroblasts [41, 456]. This all suggests that the fibrosis characteristic of ARDS may not be a late event but is switched on at a very early stage. This is particularly important as it offers the possibility for early directed treatments against fibrosis [457].

4.2.2.7. Mechanical forces and ALI

Mechanical ventilation with high volumes and pressures, and high inspired oxygen can cause structural and functional alterations in uninjured lungs, as well as enhance the damages in the injured lungs [130-133, 458]. Volutrauma is the overdistension of injured and normal alveoli. Alveolar overdistension is believed to degrade surfactant, disrupt epithelial and endothelial cell barriers, and increase cytokine levels and inflammatory cells in the lung. Elevated ventilating pressures (barotrauma) also contribute to hydrostatic alveolar flooding [458-460]. More recently, cyclic opening and closing of atelectatic alveoli during mechanical ventilation have been shown to cause lung injury independently of alveolar overdistention, by shearing of epithelial and endothelial cell layers [458, 459]. Alveolar overdistention coupled with the repeated collapse and reopening of alveoli can initiate a cascade of proinflammatory cytokines [135, 461]. The use of high FIO_2 concentration has been associated with pathologic changes in the

lung such as edema, alveolar thickening, and fibrinous exudate [462, 463]. These issues have led to a number of clinical trials of protective ventilatory strategies to reduce alveolar overdistention and increase the recruitment of atelectatic alveoli [33].

The effect of the mechanical stresses applied to the lung may extend far beyond the thoracic structures to systemic organs. Recent studies have demonstrated that mechanical ventilation can release mediators [464, 465] that may translocate into the systemic circulation [135]. This may contribute to the high prevalence of multiple organ failure in patients with ALI/ARDS. This hypothesis was strengthened by the low tidal volume study that demonstrated a systemic component in ALI/ARDS that was altered by the ventilatory strategy [33]. Injurious ventilatory strategies can lead to loss of compartmentalization in the lung and may cause translocation of mediators, endotoxin, and bacteria from the lung to the systemic circulation [138, 466]. Examination of the physiological, biological, and genetic basis of stress-induced injury to the lung in health and disease is thus a fertile area of future research.

4.3. RELEVANCE OF THE FAS/FAS LIGAND SYSTEM IN ALI/ARDS

Many lines of evidence indicates that the Fas/Fas Ligand (Fas/FasL) system plays an essential role in the pathogenesis of ALI/ARDS by inducing apoptosis and activation of inflammatory pathways in the early phase [274, 282, 284, 289, 467-473], but it also has an important role in lung repair and fibrosis [293, 295, 296, 474-477].

4.3.1. MOLECULAR BASIS OF THE FAS/FAS LIGAND SYSTEM

The Fas/Fas Ligand (Fas/FasL) system is comprised of the cell membrane surface receptor Fas (CD95) and its natural ligand, namely Fas ligand [281, 478]. Fas (CD95) is a 45-kd type I membrane receptor, a member of the tumor necrosis factor family of cell surface receptors [281, 479, 480]. FasL (CD95L), a 37-kd type II membrane glycoprotein, is also a member of the tumor necrosis factor family of cytokines [478]. Fas and FasL exist in both membrane-bound and soluble forms [481, 482]. The soluble form of FasL (sFasL) can result from enzymatic cleavage of membrane bound FasL by enzymes such as metalloproteinase-7 (MMP7, matrilysin) and metalloproteinase-3 (MMP-3) [482-488], or from rapid release by activated monocytes and monocyte-derived macrophages [471, 489-491]. Both the membrane-bound and the soluble forms of FasL are capable of inducing apoptosis of susceptible cells, and inflammation [282, 284]. Apoptosis and inflammation are induced when membrane-bound or soluble FasL binds to Fas-bearing cells [492-495]. In contrast, soluble Fas can inhibit apoptosis by binding to either membrane-bound FasL or soluble FasL, thereby preventing FasL from interacting with membrane-bound Fas receptors [496].

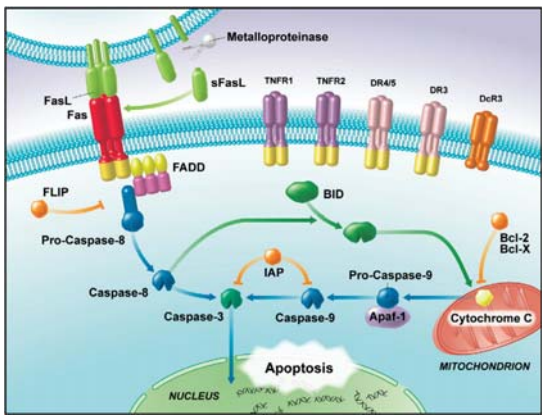
In normal lungs, Fas is expressed in myeloid cells, including neutrophils and alveolar macrophages, and also in nonmyeloid cells, including airway and alveolar epithelial cells and fibroblasts [283, 491, 497-502]. Expression in pulmonary endothelial cells has been reported to be minimal or absent [501, 503]. FasL protein is expressed in the cell membrane and cytoplasm of surface airways epithelial cells (throughout the trachea, bronchi and bronchioles), and in some alveolar type II cells. Monocytes [498], fibroblasts, neutrophils [304, 491], and lymphocytes [504-506] also express FasL protein on their surfaces. Fas and FasL proteins are co-localized in airway epithelial cells obtained from resected human lungs [502], particularly in columnar and basal cells of bronchial epithelium [468].

This coexpression of receptor and ligand is uncommon for mammalian cells in normal conditions; it has been described only for corneal epithelium, crypt cells in ulcerative colitis [507], and two hematopoietic cells, neutrophils [491], and T cells [505, 508].

Fas/FasL interactions have demonstrated a role in immune cell homeostasis. The dysregulation of the Fas apoptotic pathway contributes to several autoimmune diseases [508, 509]. However, little is known about the role of these apoptotic molecules in epithelial cells [502]. Neo- or overexpression of Fas was detected in the airway epithelium of lung inflammatory illnesses such as asthma and chronic bronchitis, as well as in the alveolar epithelium of patients with ALI/ARDS in which pro-apoptotic pathways are upregulated. It is now apparent that activation of Fas is likely to have an important role in mediating both apoptosis and inflammation in the lung epithelium. On the other hand, the expression of FasL in the lung epithelium may function to prevent infiltration of Fas-bearing inflammatory cells (e.g., eosinophils) into the epithelial layer. In that case, the FasL expression in the epithelium may confer immune privilege and protect it from the activated cell immune response as has been suggested for testis [510], brain [511], and placenta [512, 513].

4.3.1.1. The Fas/FasL system and the molecular mechanisms of apoptosis

Apoptosis is a form of regulated cell death in which activation of specific intracellular serine rich proteases (caspases) leads to DNA cleavage and cell death. Apoptosis is an essential feature of development, and provides a mechanism for tissue remodeling. Apoptosis occurs in response to activation of specific cell membrane receptors, termed “death receptors”, as well as in response to the release of mitochondrial products such as cytochrome c [514, 515] (**Figure 4.1,** adapted from T.R. Martin [241]). The death receptor family includes the TNF receptors I and II, and the Fas receptor (CD95), which is activated either by Fas ligand (FasL) on the surface of cytotoxic lymphocytes, or by a soluble form of FasL (sFasL), which can be cleaved from cell membranes by the action of specific serine proteinases, such as matrix



metalloproteinases 7 and 3 (MMP-7, MMP-3) [482-488]. Soluble FasL is also released from activated blood monocytes, but it does not appear to be released from activated alveolar macrophages [471, 489-491].

Apoptosis is initiated by the engagement and co-aggregation of FasL with the Fas receptor on the cell surface followed by a series of intracellular molecular interactions that coordinate the activation of caspases and cell death (Fig. 4.1) [516-519]. Fas is expressed on the surface of cells as pre-associated homotrimers and, upon FasL binding, undergoes a conformational change to reveal its cytoplasmic death domain (DD), which favors homotypic interactions with other DD-containing proteins. Additional interactions mediated through the 'death effector domain' (DED) of FADD with DED domains of procaspase-8 and procaspase-10 assemble the death-inducing signaling complex (DISC) [520]. Efficient DISC assembly provides a molecular scaffold which concentrates cysteine proteases in a manner that promotes autoproteolytic cleavage of caspase-8 and subsequent activation of the apoptotic pathway. Activated caspase-8 initiates the extrinsic pathway of apoptosis by converting inactive effector procaspases-3, -6, and -7 into active enzymes via transproteolysis (in type I cells), and also can activate the intrinsic pathway of apoptosis via cleavage of bcl-2 interacting domain (Bid) to generate truncated Bid that translocates to the mitochondria and induces the release of cytochrome c. Released cytochrome c activates caspace-9 that binds to Apaf-1 and activates caspase-3 (in type II cells) [519, 521, 522]. Executioner caspases cleave various vital cellular substrates including cleavage of nuclear DNA that leads to cell death. The DNA cleavage is detectable as a "laddering" effect of DNA fragments of different molecular weights when cellular DNA is analyzed by electrophoresis in agarose gels. DNA cleavage is also detectable by assays which identify nucleotide cleavage sites using a terminal deoxynucleotide transferase enzyme (TUNEL assay) [523]. Activated caspase 3, a distal enzyme in the caspase cascade, can be detected in cells and tissues using antibodies specific for the cleaved (activated) form of caspase 3.

Cellular apoptosis is tightly regulated by several different inhibitory proteins, so that cell death can be controlled at the appropriate times. These inhibitory proteins of apoptosis (IAP) directly bind and inhibit caspases 3, 6, 7, and 9 [524-526]. The mitochondrial pathway is inhibited by the Bcl-2 family of proteins, which block activation of caspases by cytochrome c [525-527]. A FLICE-like inhibitor protein (FLIP) competitively inhibits the

binding of caspase-8 to the Fas/FADD membrane complex and blocks the Fas signaling pathway [515, 528, 529] . Apoptosis leads to the loss of polarization of the cell membrane followed by the expression of phosphatidylserine on the outer leaflet of the lipid bilayer, which can be detectable using the binding of the fluorescent indicator annexin V [530]. Apoptotic cells are phagocited via recognition of the phosphatidylserine on the cell membrane surface [531].

Resolution of neutrophil mediated inflammation is achieved, in part, through induction of neutrophil apoptosis. This constitutively expressed program can be induced by ligation of the Fas receptor. However, it has been shown that activated neutrophils are resistant to pro-apoptotic signals resulting from Fas ligation [305, 532]. This resistance is associated with an increase in intracellular levels of reduced glutathione (GSH) [305] and altered caspase expression [532]. In addition, apoptosis can be delayed in neutrophils by the presence of inflammatory mediators such as insulin-like growth factor-1 (IGF1), interferon-δ (IFNG) and granulocyte–macrophage colony-stimulating factor (GM-CSF) [95]. This indicates that apoptosis of neutrophils are delayed at the site of inflammation.

In epithelial cells, one of the consequences of apoptosis is loss of cellular attachment to the underlying basement membrane. In the alveolar epithelium, this could result in the exposure of the underlying alveolar epithelial basement membrane to inflammatory products present in the alveolar spaces, such as oxidants, proteinases, and other elements of the inflammatory milieu. Destruction of the alveolar walls is also associated with the activation of fibroblast proliferation and collagen production, which could lead to fibrosis during the repair process in ALI. Delay in neutrophil apoptosis by the presence of inflammatory mediators such as insulin-like growth factor-1 (IGF1), interferon-δ (IFNγ) and granulocyte–macrophage colony-stimulating factor (GM-CSF) could also perpetuate the injury [83, 304, 473].

4.3.1.2. Fas/FasL and the molecular mechanisms of inflammation

Apoptosis has been traditionally considered a “silent” process of cell death. However, accumulating evidence indicates that apoptosis can also activate inflammatory responses. The Fas/FasL system triggers activation of a series of cysteine proteases (caspases), which leads to apoptosis. In addition, studies performed *in vitro* have established that activation of the Fas/FasL system can also lead to activation of

proinflammatory pathways including those triggered by translocation of NF-κB and ERK/AP-1 [533], and those associated with activation of the IL-1β converting enzyme (ICE, Caspase-1) [534, 535]. The intracellular signaling through the Fas pathway is regulated by the intracellular caspase-8 homolog FLICE-like inhibitory protein (cFLIP). Binding of cFLIP to the DISC can inactivate caspase-8 and shift the Fas apoptotic pathway to non-apoptotic signaling. FLIP interacts with TRAF1/RIP and with TRAF2/Raf-1, leading to NF-κB and ERK/AP-1 activation, respectively [515, 533, 536]. Activation of the transcription factors NF-κB and AP-1 [533, 536], instead of inducing apoptosis, leads to the production of proinflammatory cytokines, including neutrophil chemoattractant and factors involved in cell proliferation such as IL-2, which facilitates proliferation of T cells. Apoptosis and inflammation are not mutually exclusive; on the contrary, they seem to be intimately linked. This is particularly evident in the dual roles of some caspases (including caspase-1) in the execution of both apoptosis and the processing and secretion of pro-inflammatory cytokines (including IL-1β and IL-18). Notably, FADD and caspase-8, important molecules for the Fas/FasL system-induced apoptosis, also mediate NF-κB activation upon Fas ligation. Moreover, inactivation of caspase-8 has been associated to an increase in NF-κB activation induced by sFasL [537]. Therefore, the branch point of the Fas signal transduction pathways leading to apoptosis and NF-κB activation has been suggested to be downstream of caspase-8. However, molecular details underlying proinflammatory signaling induced by FasL are not completely understood.

In general, FasL induces inflammation by exerting a direct effect on inflammatory cells. In this sense, FasL promotes neutrophil infiltration [538] and activates macrophages. When human macrophages are activated by soluble FasL (sFasL) or the agonistic antibody CH11 *in vitro*, they do not become apoptotic, but instead release proinflammatory cytokines such as TNF-α and IL-8 associated with nuclear translocation of NF-κB [499]. In addition, soluble FasL (sFasL) activates NF-κB and ERK1/2 in serum-starved fibroblasts, leading to IL-6 and IL-8 secretion in the complete absence of cell death [537]. Interestingly, new evidence indicates that Fas/FasL mediated-inflammation is not restricted to inflammatory cells. In fact, a variety of cells including epithelial cells can also secrete cytokines and chemokines upon Fas ligation. *In vitro* studies showed that Fas ligation in bronchiolar and alveolar epithelial cells induces the secretion of the neutrophil chemoattractant IL-8 through NF-κB activation in combination with activation of pro-apoptotic pathways [539]. In human kidney cells

(HEK293 cells), FasL induces cell-autonomous NF-κB activation and IL-8 production without inducing detectable apoptosis [540]. Vascular smooth muscle cells upregulated a number of cytokines and chemokines in response to Fas ligation, including IL-8, IL-1α, MCP-1, GRO-1 and IL-6. Therefore, locally excessive FasL-mediated apoptosis is not a 'silent' process but it can promote secretion of proinflammatory cytokines and recruitment of inflammatory cells. This double function may facilitate cytokine-mediated recruitment of inflammatory phagocytes to clear apoptotic bodies from areas of excessive apoptosis, but cytokines and active phagocytic cells have also been directly implicated in the generation of local tissue damage [469, 473].

4.3.2. RELEVANCE OF FAS/FAS LIGAND SYSTEM IN ALI

4.3.2.1. Clinical studies

Albertine et al. found that, in the lungs of patients with ALI or ARDS, both Fas and FasL are co-expressed by alveolar epithelium and distal airway as well as by inflammatory cells (neutrophils and macrophages/monocytes) located in the air spaces [468]. The local up-regulation of Fas/FasL is greater in the lungs of patients who died, and is associated with greater apoptosis of cells in the alveolar wall [468]. S. Hashimoto et al. also found up-regulation of mRNA for a number of molecules associated with apoptosis, including Fas and FasL, in the cellular component of the BAL fluid from patients with septic ARDS [467].

G. Matute-Bello and T.R. Matin (mentors) first found that soluble Fas ligand is present in the BAL fluid in humans with early ARDS, and reaches higher concentrations in the lung fluids from patients who die. The soluble Fas ligand present in the lung fluids from patients with ARDS is biologically active and can induce apoptosis in normal human distal lung epithelial cells [284]. Albertine et al. confirmed these findings by detecting higher concentrations of soluble FasL in the undiluted pulmonary edema fluid of patients with ALI or ARDS compared to control patients with hydrostatic pulmonary edema. In addition, the concentration of soluble FasL was significantly higher in the pulmonary edema fluid of patients with ALI or ARDS compared to simultaneous plasma samples, indicating local production in the alveolar compartment [468].

Matute-Bello and Martin also demonstrated that cells recovered from the airspaces of patients with ARDS release sFasL (40), which increased after stimulation with lipopolysaccharide (LPS) [284]. This can be particularly relevant since LPS is also present in the BAL fluid of many patients with ARDS [357, 358]. A separate group determined that FasL mRNA was increased in the cellular component of BAL fluid retrieved from patients with early septic ARDS [468]. These results show that sFasL is produced or released locally in the airspace compartment. The alveolar macrophages are a possible source, since cells of the human monocyte/macrophage lineage contain FasL and release it when activated by phytohemagglutinin, immune complexes, or superantigen [471, 490, 541]. A second possibility could be that sFasL increases in the circulation as part of the systemic inflammatory response, and then moves into the airspaces when endothelial and epithelial permeability increases. Indeed, sFasL concentration was not only elevated in the BAL fluid but also in serum of patients with ARDS and other pulmonary inflammatory diseases, such as idiopathic pulmonary fibrosis [295], bronchiolitis obliterans-organizing pneumonia [240], hypersensitivity pneumonitis, and pneumococcal pneumonia [542]. In addition, plasma sFasL was increased in patients with ARDS who underwent ventilation with a conventional strategy compared with the lung protective strategy, and it was associated with the development of renal dysfunction [543]. Elevated concentrations of sFasL have also been found in serum of patients with cardiopulmonary arrest [544] and chronic renal failure [545]. Thus, it is possible that both locally shed sFasL and systemic influx of circulating sFasL account for the increased concentrations that were detected in BAL fluid from patients with ARDS. The elevated concentrations of sFasL in either BALF or serum were associated with an increase in the progression to multiple organ failure and mortality in ARDS [284, 546, 547].

Taken together, these findings demonstrate that the Fas/FasL system is upregulated in the lungs of patients with acute lung injury and that sFasL is released into the airspaces in vivo during lung injury as a biologically active molecule capable of inducing apoptosis in Fas-susceptible target cells of the lungs. Altogether, these studies suggest that the Fas/FasL system is likely to have a role in the pathogenesis of the distal lung epithelial injury seen in ALI or ARDS.

4.3.2.2. Animal studies

Animal studies have provided evidence supporting a key role for the Fas/FasL system in lung disease. Fas is detectable on airway epithelial cell (Clara cells) and alveolar type II cells in rodents [280, 283, 502], and the expression of Fas in epithelial cells increases in response to inflammatory mediators such as lipopolysaccharide (LPS) [274]. We have shown that activation of the Fas/FasL system by either a Fas-activating mAb or recombinant human sFasL causes ALI and lung epithelial apoptosis in mice and rabbits [548, 549]. Fine et al. also found that activation of Fas caused apoptosis of murine alveolar type II cells *in vivo* [283]. In a murine model of LPS-induced lung injury, the functional Fas/FasL system is required for the alveolar wall permeability changes and inflammation, as Fas-deficient mice had significantly less lung injury [274]. Fas- and Fas ligand–deficient mice showed marked protection from lung inflammation and apoptosis and decreased severity of lung injury in models of ALI mediated by sepsis after cecal ligation and puncture [550]. Other studies also showed that Fas-deficient mice had significantly reduced mortality from hypoxia-induced lung injury during *Legionella* pneumonia [551] or after challenge with intrapulmonary deposition of the IgG immune complexes [472]. In addition, activation of the Fas/FasL system has also been associated with development of distant organ failures (e.g. renal dysfunction) in models of injurious mechanical ventilation [543]. Thus, a growing body of evidence from studies of humans and animal models links activation of the Fas/FasL system with lung injury. However, how activation of Fas/FasL contributes to the development of injury in the lungs is currently unknown.

4.3.3. MECHANISMS OF LUNG INJURY BY THE FAS/FAS LIGAND SYSTEM

4.3.3.1. Fas/FasL system and apoptosis in alveolar epithelial cells in ALI

Damage to alveolar epithelial cells is one of the earliest events that occur in the development of ALI/ARDS in humans, and the severity of epithelial damage is associated with morbidity and mortality [18, 38, 106, 241, 258, 552]. The actual mechanisms leading to alveolar epithelial damage remain unclear, but are likely to involve a combination of necrosis and apoptosis. Necrosis of alveolar epithelial cells has been found in the lungs of patients who died with ALI/ARDS, and is thought to be caused

directly by mechanical factors, local ischemia, or bacterial products in the airspaces [85, 110, 259, 553]. Apoptotic cells have also been found in the alveolar wall of these patients. Activation of Fas in the cell membrane is one of the most potent mechanisms to induce cellular death by apoptosis [241, 258, 284, 468, 470, 554, 555].

4.3.3.1.1. Clinical studies

Open lung biopsies performed on ARDS patients reveal apoptosis in Type II pneumocytes [110, 272]. The alveolar epithelium from humans with diffuse alveolar damage shows increased expression of the apoptotic promoter Bax, a homolog of Bcl-2 [273]. Albertine et al. also reported increase in markers of apoptosis (terminal dUTP nick-end labeling, capase 3, Bax, and P53) in the alveolar wall of patients who died with ALI or ARDS as compared with patients who died without pulmonary disease. In addition, both Fas and FasL have been shown to be co-expressed by epithelial cells in the alveolar wall, as well as, inflammatory cells located in the airspaces, and to a greater extent in patients who died with ALI or ARDS compared with those dying without pulmonary disease. The local up-regulation of the Fas/FasL system occurred in the alveolar epithelium and was associated with greater apoptosis and worse clinical outcome in patients with ALI/ARDS [468].

T.R. Martin's group found that human distal lung epithelial cells are sensitive to sFasL-induced apoptosis, whereas proximal lung epithelial cells are relatively insensitive [497]. These studies raise the possibility that there is a gradient of Fas sensitivity in the lungs, with the proximal airways relatively insensitive to sFasL and increasing sensitivity moving distally in the airways and into the alveolar space. The mechanisms for the differential sensitivity of proximal and distal pneumocytes to sFasL remain unclear, as both types of cells express membrane Fas and contain similar patterns of steady-state mRNAs for the Fas pathway intermediate proteins. These findings are important since epithelial damage occurs primarily in distal airway and alveolar epithelium in ALI/ARDS, whereas sFasL is present throughout the airspaces [497]. Altogether, these findings show that in humans with ALI or ARDS, the Fas/FasL system-induced apoptosis seems to be an important mechanism in alveolar epithelial injury [468, 556].

4.3.3.1.2. Experimental studies

In animal models, T.R. Martin's group found that intratracheal instillation of recombinant human sFasL causes lung injury and epithelial apoptosis in the lung of rabbits [549,] , and that activation of Fas using an activating monoclonal antibody (Jo-2), causes injury and epithelial apoptosis in the lungs of mice [555] . Rabbit and mouse alveolar type II cells are sensitive to Fas activation either by human sFasL (in rabbit pneumocytes) or by a specific monoclonal antibody, which clusters membrane Fas (in murine pneumocytes) [549, 555]. Furthermore, the absence of a functional Fas–FasL system in mice or the administration of Fas-blocking antibodies decrease the severity of ALI and diminishes epithelial cell apoptosis and pulmonary inflammation [282, 284, 472, 555]. These collective data suggest that the Fas/FasL pathway may play an important role in apoptosis of alveolar epithelial cells and disruption of the alveolar-epithelial barrier in ALI.

4.3.3.1.3. Modulatory factors of Fas-mediated apoptosis in alveolar epithelial cells

Several factors modulate Fas-mediated apoptosis of alveolar epithelial cells. Surfactant protein A (SP-A), the primary protein present in pulmonary surfactant, is an inhibitor of type II apoptosis *in vivo* [287, 288]. This is important because in patients with early ARDS, the concentration of SP-A is decreased in BAL fluid [214]. The lower concentration of SP-A would favor apoptosis of Type II cells in these patients. Another important modulator of Fas ligand in the lungs is angiotensin II. Epithelial cells interact with angiotensin II via the angiotensin receptor subtype AT1, and this interaction is required for Fas-mediated apoptosis of alveolar epithelial cells *in vitro* [268]. Fas ligand and angiotensin II can induce apoptosis in cell lines derived from Type II cells [268, 285]. In ARDS the concentration of angiotensin-converting enzyme, which catabolizes the conversion of angiotensin I to angiotensin II, is increased in BAL fluid [286]. Therefore, in early ARDS a combination of three factors favors alveolar epithelial apoptosis: increased concentrations of soluble Fas ligand; decreased concentrations of SP-A; and increased concentrations of angiotensin-converting enzyme and angiotensin II. Together, these studies suggest that the Fas/FasL system may mediate, in part, the injury to distal lung epithelial cells that occurs in patients with ALI or ARDS.

4.3.3.2. Fas/FasL system and inflammation in ALI

It was recently suggested that Fas (CD95) signaling can have apoptotic or pro-inflammatory functions, and that the resultant effects strongly depend on the cellular microenvironment [469]. The role of the proinflammatory function of the Fas/FasL system in the pathogenesis of lung injury remains unclear. In experimental studies, activation of the Fas/FasL system in the lungs consistently leads to activation of inflammatory responses characterized by cytokine release and neutrophil recruitment into the lungs [499, 557-559]. Wortinger et al. found that instillation of recombinant FasL into the lung induces production of KC and MIP-2 and the recruitment of neutrophils into the lung in animal models [560]. Furthermore, G. Matute-Bello and T.R. Martin (mentor group) reported that Fas activation *in vivo* resulted in acute lung epithelial injury, lung inflammation, and recruitment of neutrophils [282, 548, 549, 555] and that Fas also modulated LPS-induced lung inflammation [274, 561]. The relevance of the proinflammatory function of the Fas/FasL system was confirmed in studies by Neff et al. who demonstrated that mice deficient in Fas have an impaired neutrophilic response and less production of KC and MIP-2 in response to inhaled LPS [472, 562]. In addition, silencing of Fas in the lungs protected against lung inflammation in models of hemorrhagic shock and septic shock followed by cecal ligation and puncture [563]. Furthermore, blockade of the Fas/FasL system by specific pharmacological inhibitors or Fas mutation (*lpr* mice) resulted in reduced neutrophil counts in the bronchoalveolar lavage (BAL) fluid and lower concentrations of TNF-α and MIP-2 at 48 h after intratracheal instillation of *S. pneumoniae* [564]. Together, these studies suggest that the Fas/FasL system may play an important role not only in apoptosis but also in the development of an inflammatory response in the lungs following exposure to LPS, live bacteria, and sepsis.

In *in vitro* experiments, Fas ligation of human monocyte-derived macrophages resulted in the release of pro-inflammatory factors such as TNF-α and IL-8. Murine alveolar macrophages release KC and MIP-2 in response to human recombinant soluble FasL [490, 499]. Activated monocytes and macrophages by Fas ligation did not become apoptotic. In contrast, A.M. Hohlbaum et al. determined that stimulation of peritoneal neutrophils released pro-inflammatory factors (MIP-2), and at the same time, these cells underwent cell manifestations of apoptosis [565]. In addition, the apoptotic death of this cell population in the peritoneum preceded neutrophil inflammation, whereas the soluble form of FasL (sFasL) did not induce apoptosis as mentioned above.

Interestingly, bronchiolar epithelial cells undergo apoptosis and also secrete IL-8 by activation of nuclear factor-kappa B (NK-κB) in response to Fas ligation *in vitro* [539]. Current studies determined that the murine alveolar epithelial cell line MLE-12 releases the neutrophil chemoattractant KC, MIP-2 and MCP-1 in response to Fas activation *in vitro* [566], which was abrogated by inhibiting ERK1/2 signaling [550, 567]. Therefore, it could be possible that FasL signaling induces inflammatory apoptosis in epithelial cells and alveolar macrophages, with concomitant cytokine and chemokine release leading to neutrophil infiltration, which in turn may amplify the inflammatory cascade in lung injury.

Activation of pro-inflammatory pathways in both lung epithelial cells and macrophages by Fas ligation raises the question of whether the neutrophilic inflammatory response mediated by Fas in lungs is due to direct activation of alveolar macrophages or instead is secondary to an initial effect on lung epithelial cells. To answer this question, T.R. Martin's group used chimeric mice lacking Fas in either myeloid or non-myeloid cells [555]. They determined that mice expressing Fas only in their myeloid cells (alveolar macrophages) showed little response to Fas activation, whereas the mice expressing Fas in their non-myeloid cells (lung epithelium) showed evidence of both inflammation and apoptosis, suggesting that alveolar macrophages are not required for the development of Fas-induced lung inflammation in mice [555]. This was also confirmed in macrophage-depleted mice which developed a neutrophilic inflammatory response following Fas activation *in vivo*, suggesting that other cell type(s) in the lung can promote inflammation [566]. In this sense, the alveolar epithelium may be an important source of pro-inflammatory cytokines during the early phase of lung injury [555, 566, 568-573]. To support this notion, some studies have shown that primary human alveolar Type II cells stimulated with LPS release chemokines (MCP-1, GRO, and IL-8) [571]. Altogether, these results suggest that the lung neutrophilic inflammatory response to Fas activation is not primarily dependent on alveolar macrophages and may depend instead on cytokine release by alveolar epithelial cells. Also, it may be possible that this inflammatory response is favored by secondary activation of inflammatory cells in response to phagocytosis of apoptotic epithelial cells.

4.3.3.3. Fas/FasL system and protein permeability

Acute lung injury (ALI) and acute respiratory distress syndrome (ARDS) are characterized by increased protein permeability across the pulmonary microvascular

endothelium and alveolar epithelium [1, 210]. The main structural manifestation of increased protein permeability is the disruption of alveolar epithelial cells [85, 108, 237, 574]. The mechanisms by which the alveolar epithelium is injured in humans with ALI or ARDS are not totally clear. Nevertheless, some studies indicate that Fas-mediated apoptosis is one of the mechanisms involved in alveolar epithelial damage and consequently in the alteration of the alveolar protein permeability epithelial apoptosis [284, 468, 555].

In animal models, G. Matute-Bello and T.R. Martin also determined that Fas-mediated lung injury requires expression of Fas expression on nonmyeloid cells including lung epithelial cells, and is associated with apoptosis of cells of the alveolar walls, increased alveolar permeability, and moderate neutrophilic inflammation in the absence of cytokine expression (MIP-2, KC). These findings suggest that the alveolar epithelium is the primary target of Fas-mediated acute lung injury [555]. Furthermore, the neutrophilic response in the mice expressing Fas in myeloid cells (alveolar macrophages/monocytes and neutrophils) was small and was not associated with permeability changes. This suggests that in those animal models of lung injury mediated by Fas activation, the increase in protein permeability is due to Fas-mediated injury in the epithelium, rather than by inducing an inflammatory response.

4.3.3.4. Fas/FasL system and pulmonary fibrosis

The control of cell number is determined by a balance between cell proliferation and cell death. Apoptosis, then, may have a role in human diseases in two different ways. Increased apoptosis in the early phase of ALI, particularly in alveolar epithelial cells, may contribute to respiratory failure. However, apoptosis can also have a beneficial role in lung repair in the proliferative and chronic phase of ALI when the elimination of proliferating mesenchymal and inflammatory cells from the alveolar air space and the alveolar walls are required [575, 576]. Excessive alveolar epithelial cell death could overwhelm the necessary elimination of proliferating mesenchymal and inflammatory cells after the early phase of lung injury. This could prolong the inflammatory response [576, 577] and interfere with re-epithelization, which leads to overgrowth of lung fibroblasts and development of lung fibrosis [238, 576].

4.3.3.4.1. Fibrosis in ALI

The early inflammatory response in ALI/ARDS, which is maximal during the first three days, is then followed by a fibroproliferative response [38] [1, 210]. The fibroproliferative response is thought to occur late in the course of ALI/ARDS, but autopsy studies have shown histopathological evidence of fibroproliferation as early as day 5 with evidence of collagen production in the lungs even on the first day of ALI/ARDS [83, 112, 400, 578, 579]. The development of a fibroproliferative response has been repeatedly identified as an independent poor prognostic factor in ALI/ARDS [84, 448, 580].

4.3.3.4.2. Lung fibrosis and apoptosis of lung epithelial cells

A prominent feature of lung fibrosis is the proliferation and accumulation of mesenchymal cells, which include abnormal fibroblast populations that emerge in the interstitium but may migrate into the alveolar spaces [581-584], and endothelial cells that proliferate during the neovascularization of nascent fibrotic foci [575, 576]. Apoptosis has been identified as an important mechanism to remove excess cells to recover from lung fibrosis. On the other hand, apoptosis of epithelial cells has been reported to contribute directly to the pathogenesis of lung fibrosis. Epithelial apoptosis occurs in regions of the heaviest myofibroblast activity and collagen accumulation in patients with idiopathic pulmonary fibrosis (IPF) or chronic hypersensitivity pneumonitis. Apoptosis can damage the lung epithelium, which has important antifibrotic functions, including: 1) constitutive synthesis of inhibitors of lung fibroblast proliferation such as prostaglandin E2; 2) the synthesis of urokinase-type plasminogen activator and the degradation of fibrin exuded from damaged capillaries; 3) the provision of a physical barrier that protects underlying interstitial cells from mitogens that are released by activated alveolar macrophages [239, 241, 294, 582, 583, 585]. Therefore, apoptosis results in loss of the alveolar epithelial cells and their normal antifibrotic functions, which may directly promote proliferation of lung fibroblasts and collagen deposition [241, 294, 296, 582, 583, 586, 587].

4.3.3.4.3. Lung Fibrosis and Fas/FasL system

a) Clinical studies

In the lung tissue from patients with idiopathic pulmonary fibrosis, the expression of Fas and FasL proteins was upregulated in bronchiolar and alveolar epithelial cells and in infiltrating lymphocytes or granulocytes, respectively indicating a potential role of the Fas/FasL system in the development of lung fibrosis. The Fas-FasL pathway has been shown to contribute to severe epithelial damage and seems to be a predisposing factor for fibroblast proliferation in ARDS [240, 295]. The soluble FasL released into the air spaces is biologically active and induces apoptosis of epithelial cells during acute lung injury [284]. Alveolar epithelial damage in humans with ALI or ARDS is in part associated with the local upregulation of the Fas-FasL pathway and activation of the apoptotic cascade in epithelial cells [468]. The Fas/FasL system is also upregulated in lung epithelial cells and infiltrating inflammatory cells in lung tissues from patients with idiopathic pulmonary fibrosis (IPF) [240, 295]. In these patients, the expression of FasL is increased in myofibroblasts. The BAL fluid from patients with ARDS or IPF could induce apoptosis in small airway epithelial cells which are sensitive to the Fas-FasL pathway [284, 295, 476]. FasL molecules are reported to be expressed on α-smooth muscle actin positive cells in mice with bleomycin-induced pulmonary fibrosis, and in humans with IPF [469, 473, 476]. Inhibiting this pathway may be one of the novel treatment strategies against lung injury and fibrosis.

b) Experimental studies

Activation of Fas receptor in the lungs is associated with apoptosis of bronchial and alveolar epithelial cells and acute lung inflammation followed by delayed fibrosis in mice [238, 241, 296]. Also, the induction of lung fibrosis by intratracheal instillation of bleomycin is associated with the up-regulation of Fas on the epithelium associated to epithelial apoptosis that preceded the accumulation of collagens [293, 295]. Blockade of the Fas receptor by administration of a Fas-soluble form of Fas antigen and anti-FasL antibody prevented the development of bleomycin-induced fibrosis, and Fas- and FasL-deficient mice were resistant to the induction of pulmonary fibrosis [295, 588]. This suggests that Fas-induced apoptosis plays an essential role in the development of the fibrotic response [295, 469, 473]. G. Matute-Bello showed that in mouse lungs, repeated

activation of Fas resulted in apoptosis of bronchial and alveolar epithelial cells, but not fibroblasts, which subsequently led to pulmonary fibrosis in mice [238]. These results imply that the resistance of fibroblasts to Fas-mediated apoptosis may be associated with the pathophysiology of pulmonary fibrosis. In animal models of bleomycin-induced lung fibrosis there is an increase of FasL in myofibroblasts as occurs in the lungs of patients with IPF. It has been determined that FasL-expressing myofibroblasts may act as effector cells to induce Fas-dependent epithelial cell apoptosis during lung fibrosis. This indicates that myofibroblasts may prolong the epithelial cell apoptosis, which could impair the re-epithelialization in lung fibrosis. Several other studies have indicated the existence of "cross-talk" between lung epithelium and myofibroblasts, which may be involved in the development of lung fibrosis [589]. Epithelial cells overlying fibroblastic foci were found to be apoptotic [590], and fibroblasts isolated from fibrotic human or rat lungs were shown to produce factors capable of inducing epithelial cell apoptosis [589].

Fas ligation over time induces not only apoptosis but also inflammatory responses and pro-fibrotic factors such as TGF-β. Fas activation in the lungs has previously been shown to upregulate TGF-β expression, and several studies have suggested that TGF-β plays an important role in the development of fibrotic responses in mice [591]. More recently, a study by G. Matute-Bello found that activation of the Fas receptor pathway in the lungs is associated with up-regulation of metalloproteinase-12 (MMP-12) in alveolar macrophages [296, 365, 592] and that deficiency in MMP-12 protected from Fas-mediated fibrosis. It has been found in *in vitro* studies that MMP-12 has a wide variety of potential substrates, including type IV collagen, fibronectin, laminin, and gelatin, as well as non-matrix proteins such as α1-antitrypsin and latent TNF-α [593]. The mechanism by which MMP-12 has a role in Fas-mediated lung fibrosis is not yet clear. One possibility is that Fas dependent apoptosis of alveolar epithelial cells exposes the alveolar basement membrane and allows MMP-12 and other metalloproteinases to degrade the matrix, triggering a fibroproliferative response [296].

4.3.4. CONTROL OF THE BIOLOGICAL ACTIVITY OF sFasL

4.3.4.1. FasL structure

FasL protein is expressed in two distinct forms: 1) a membrane form on the cell surface or stored in intracellular microvesicles (mFasL), which are excreted into the intercellular milieu in response to various physiologic stimuli; and 2) a soluble form (sFasL) generated by the cleavage of membrane FasL by matrix metalloproteinases expressed on the cell surface [482-488] or released by activated monocytes [471, 489-491].

The membrane FasL is a 40 kDa type II protein that acts locally through cell-to-cell contact. The cell membrane form of FasL comprises cytoplasmic, transmembrane and extracellular regions. As with other members of TNF family, the extracellular domain of FasL can be cleaved by matrix metalloproteinases into a 26-29 kDa soluble form (sFasL). The extracellular domain is composed of the stalk region (103-136 aa) at the NH_2-terminus (closer to the cell membrane) and the TNF homology domain (137-281aa) at the COOH-terminus. It has been identified a self assembly (SA) region spanning aa 137 to 183 of the FasL ectodomain. The oligomerization of mFasL seems to be essentially dependent on the SA region and is required for activation of Fas and intracellular signalling. Membrane-bound FasL presumably exhibits its biological activity as a homotrimeric complex. The TNF homology domain (THD) expresses the highest similarity to related proteins, but also mediates highly specific binding to the cysteine-rich domains of the Fas receptor [594-596]. The cleavage site of FasL is outside the regions that are required for multimerization and receptor binding. That means that sFasL carries both domains and theoretically could have the capacity to form multimers and bind to Fas. In fact, human sFasL has also been shown to form trimers in *in vitro* studies.

4.3.4.2. Functional activity of sFasL

There is substantial controversy in the literature over the physiological role of sFasL. Some studies found that sFasL in solution was less effective than membrane FasL in inducing apoptosis, and that sFasL inhibited the cytotoxicity mediated by membrane FasL [482, 597, 598]. Thus, it has been proposed that sFasL might function to protect healthy bystander cells bearing FasL, such as cytotoxic T cells [565]. In some studies,

neither human nor murine sFasL could induce apoptosis in cultured lymphocytes, hepatocytes, or colon carcinoma cells [565]. On the other hand, human sFasL induced hepatic failure in mice that had been pretreated with bacteria, whereas mice deficient in Fas receptor (lpr mice) were not affected [599], indicating that human sFasL induced liver damage by Fas activation. T.R. Martin's group demonstrated that intratracheal instillation of human sFasL induced lung injury in rabbits. In accordance with that, sFasL is also pro-apoptotic *in vitro* against Helicobacter pylori-infected gastric epithelial cells, mouse hepatocytes and lymphoma cells among others. Furthermore, two separate groups also found that neutrophils undergoing apoptosis release sFasL capable of inducing cell death of lung epithelial cells [577] and Jurkat cells [491]. On the other hand, it has been suggested that sFasL can activate inflammatory responses rather than apoptosis. Human sFasL can recruit human and mouse PMN *in vitro* [538, 600] and reduces neutrophil adhesion to endothelial cells [601, 602]. Therefore, it seems that sFasL can have proapoptotic, antiapoptotic, and neutrophil recruitment functions depending on the nature of other contextual mediators in the microenvironment [554, 597, 600, 603-606].

The difference between the membranous and the soluble FasL in activating apoptosis is not completely understood. The receptor Fas needs to co-aggregate into high molecular weight clusters in order to transmit the intracellular death signal [518, 519]. Some studies suggest that the membrane-bound FasL has capability to form trimers and larger aggregates on the cell surface that facilitates the formation of the receptor Fas clusters which results in oligomerization of a cytoplasmic death domain (DISC) and, consequently, intracellular death signalling [518, 519, 521, 607, 608]. Although sFasL exits as a homotrimer and binds to Fas, some studies show that it is ineffective in forming Fas clusters necessary to activate apoptotic signal, but is capable of activating non-apoptotic signals [519, 540, 554, 605, 609, 610]. Thus, the transduction of the apoptotic signal by membranous FasL can be blocked in the presence of sFasL and the observed overall effect is inhibition of apoptosis [597, 598, 611]. Another possibility for the differences in the activity between membranous and soluble forms of FasL is that accessory molecules may act to enhance the activity of membrane FasL. S. Sieg et al. determined that the expression of ICAM-1 on the cell surface with FasL enhances the capacity of FasL to induce apoptosis [612]. The sFasL, however, can induce apoptosis following association or aggregation with extracellular matrix proteins [613, 614] when cells overexpress Fas on

their surface [481, 598] or when engineered to form tetramers and higher order structures [615-617].

The discrepancies among studies may be due to the presence of accessory molecules that may enhance the function of sFasL, the type of tissues and cells used to test cytotoxicity and the environmental conditions. In addition, these differences may be explained by the different methods to generate sFasL and the use of sFasL from distinct species (human vs mouse) and with different N-terminal sequences.

4.3.4.2.1. Cleavage sites

Different cleavage sites have been identified in the extracellular domain of FasL where several matrix metalloproteinases (MMPs) can act to generate sFasL[483, 484, 487, 488, 597, 618]. The multiple forms of sFasL that have been used so far had different N-terminal sequences, which may represent alternative proteolytic cleavage products. Tanaka el al. reported that natural cleave short form of mouse sFasL (amino acids 127–279) was not cytotoxic [482]. In contrast, Berg and Suda showed that a recombinant protein corresponding to the entire extracellular domain of mouse FasL (amino acids 101–279) induced apoptosis [619, 620].

Recent studies have shown that matrix metalloproteinase 7 and 3 (MMP7-3) cleave FasL in certain cell types [488]. In contrast to the previously described sFasL fragments, the soluble protein generated by MMP7 cleavage has pro-apoptotic activity *in vitro* in prostate epithelial cells and *in vivo* [487, 621]. Therefore, it is possible that differential proteolysis of FasL could yield soluble forms with unique biological activities. This could explain in part the discrepancies related to the function of sFasL [488].

Three different cleavage sites in FasL have been reported for MMP-7: 1) ELAELR present in the stalk region close to the cell membrane, 2) SL, upstream to the previous one, also present in the stalk region, and 3) ELR, the closest to the C-terminus, present in the trimerization domain within the TNF domain. It is possible that processing at the ELAELR site occurs first, followed by a secondary cleavage at the C-terminally located SL sites in both murine and human FasL [488]. In other studies, cleaved sFasL at the trimerization domain created a sFasL molecule that was no longer active [622]. Therefore, it is possible that the existence of different cleavage sites of FasL may yield

a variety of soluble forms with different biological activities, which also implies that MMPs such as MMP-7 could be a mechanism to regulate the activity of FasL *in vivo*.

4.3.4.2.2. Aggregation

Numerous proteins can form multimers composed of two or more polypeptide chains which may be alike (homo-multimers protein) or different (hetero-multimers protein). The degree of multimerization has been proposed to tightly control the biological activity of some proteins by diminishing or enhancing their activity. Also, it has been observed that multimeric proteins normally are more resistant to degradation [623, 624].

As mentioned before, the membrane-bound FasL has the capability to form aggregates of higher molecular weight while binding to receptor Fas [625]. Formation of high molecular weight Fas-FasL complexes results in oligomerization of a cytoplasmic death domain (DISC) and, consequently, intracellular death signal transmission [522, 626]. Some studies have found that human sFasL also forms trimers but that these trimers cannot initiate the apoptosis signal [611]. Two separate groups artificially aggregated human sFasL units upon addition of cross-linking antibodies and observed that this larger molecule of sFasL restored its cytotoxic activity in *in vivo* and *in vitro* models [597, 615]. P. Schneider et al. compared the effect on mouse liver of this cross-linked aggregated sFasL to its non-aggregated form. The cross-linked sFasL induced lethal liver hemorrhages and apoptosis in hepatocytes, whereas the non-aggregated sFasL treated hepatocytes had a normal morphology [597]. Moreover, Holler et al. showed that both trimeric or hexameric sFasL bound to Fas, but only the hexameric forms were highly cytotoxic and competent to signal apoptosis via formation of death-inducing signaling complex [615]. They suggested that a minimal multimerization into hexamers of sFasL is required to trigger the intracellular death signal.

It has been reported that a substitution of F275 to L275 in human FasL mice leads to an inadequate oligomerization of FasL molecule along with a significant loss of activity [622, 627]. However, substitution of amino acid residues located at the binding site resulted in a deficiency in Fas-FasL binding and a significant loss of cytotoxicity but its capacity to multimerize was preserved. This suggests that both multimerization and adequate binding of FasL to its receptor are needed in order to preserve its cytotoxic activity.

4.3.4.2.3. Level of Fas expression in the cell membrane and type of cell

It also has been proposed that the activity of sFasL is target-cell specific. Some cells have different sensitivities to the soluble and the membrane-bound forms of sFasL. Although sFasL has less capability to induce apoptosis than the membrane-bound FasL, some cells can be killed by sFasL under certain circumstances. For example, mouse W4 and hFasL/ WR19L cells that overexpress Fas could be efficiently killed by natural cleaved human sFasL [483, 492]. In contrast, human Jurkat cells or mouse primary hepatocytes that express a moderate level of Fas endogenously were resistant to human sFasL but were efficiently killed by membrane-bound FasL [281, 597, 612, 622]. Therefore, the levels of the receptor Fas expressed in the cell surface can explain in part the differences in the sensitivity to sFasL. Also the use of tumor cells verus non-tumor cells, lymphoid cells vs epithelial cells, and transformed cells vs primary cells that display different behaviors can also account for the different activity of sFasL found in the literature.

4.3.4.2.4. Species-specific differences

Species-specific differences have been detected in the activity of sFasL. Some studies show that mouse sFasL is less cytotoxic than human sFasL *in vitro* [619]. In our preliminary animal experiments, we confirmed that intratracheal instillation of mouse sFasL into mouse lungs was poorly cytotoxic, whereas instillation of human sFasL resulted in the development of lung injury *in vivo*.

4.3.4.2.5. Accessory molecules

Association of proteins to other molecules is a general phenomenon that controls protein activity. For instance, immunoglobulins associate with many proteins in the lungs, which leads to important changes in activity those proteins. In particular, the cell adhesion molecule ICAM-1 has been shown to enhance the activity of FasL *in vitro*, which raises the possibility that sFasL activity might be also regulated by associations with other molecules in the lungs [612]. In addition, membrane-bound FasL has been found to bind to fibronectin (FN) and vitronectin, two prototypic cell-adhesive glycoproteins of the extracellular matrix (ECM), and the resulting ECM-anchored FasL retains its cytotoxic potential [613, 614]. The soluble form of FasL can also bind to these

extracellular matrix products, resulting in the recovery of cytotoxic activity. The sFasL can induce apoptosis when it is anchored to the extracellular matrix to form oligomers [613, 614]. In contrast, if sFasL is released in a milieu where the formation of oligomers is unattainable, it may exert an antiapoptotic effect by competing with the membranous form for binding to Fas [598, 603, 625, 628]. Therefore, sFasL may have either apoptotic or antiapoptotic attributes depending on the characteristics of the extracellular matrix.

4.3.4.2.6. Glycosylation

Glycosylation occurs in many proteins at the end of the protein synthesis process in the Golgi apparatus. Although this phenomenon can alter the bioactivity of some molecules, FasL bioactivity does not seem to be affected. Human FasL contains within its ectodomain three N-glycosylation sites (Asn-184, Asn-250, Asn-260) which appear to be required for efficient intracellular posttranscriptional trafficking and secretion of sFasL protein outside the cell. Amino acid replacement at these N-glycosylation sites significantly reduces FasL protein production but does not interfere in its activity. This is supported by the fact that the three amino acids involved in glycosylation are located far from the interface area between FasL and Fas, so the capacity of FasL to bind to Fas seems to be preserved [594, 595, 629].

5. – HYPOTHESIS AND OBJECTIVES

Acute Lung Injury (ALI) and its severe form, the Acute Respiratory Distress Syndrome (ARDS), are a frequent complication and cause of mortality in critically ill patients. A major feature of ALI that influences its severity and outcome is the development of alveolar epithelial cell injury, in which apoptosis is known to play a significant role.

Activation of the Fas receptor/Fas ligand system is thought to be a major mechanism responsible for apoptosis of alveolar epithelial cells in humans with ALI and animal models of acute lung injury. FasL exists in membrane and soluble forms, both of which can bind and cluster the Fas receptor on the cell surface of target cells, initiating intracellular apoptotic and inflammatory signals. The soluble form of FasL can be generated from the cleavage of the membrane FasL by the proteolytic action of metalloproteinases (MMPs). This soluble FasL (sFasL) is known to increase in lung edema fluid of patients before and after ALI/ARDS, but only the sFasL present in the BAL fluid from patients with established ALI/ARDS induces apoptosis in human distal lung epithelial cells *in vitro*. This observation suggests the existence of mechanisms that modulate the bioactivity of sFasL *in vivo*.

The mechanisms responsible for the biological activity of sFasL *in vivo* are not completely understood. Three cleavage sites have been identified in the extracellular domain of the membrane FasL that give rise to variants of sFasL with different N-terminal sequences and different biological activities. Several lines of evidence suggest that the degree of aggregation is also an important determinant of the biological activity of sFasL. Oxidation of certain amino acid residues, such as methionine, has been shown to modify the degree of aggregation and bioactivity of some proteins. The alveolar environment in patients with ALI contains abundant oxidants. The generation of reactive oxygen species by activated neutrophils in the lungs may lead to structural and functional modifications of many proteins, including sFasL.

HYPOTHESES

In the present work, we investigated the following hypotheses:

1. The biological activity of sFasL in vivo depends on the extent of proteolytic cleavage, and the corresponding length of the N-terminal sequence.
2. The oxidation of methionine residues in sFasL promotes aggregation of sFasL and modifies its bioactivity in the lungs of patients with ARDS.

OBJECTIVES

1. To identify the intrinsic structural determinants that modulate the biological activity of human sFasL:
   a. To determine whether the presence of the stalk region at the N-terminus of sFasL influences its biological activity in vivo, and
   b. To determine whether metalloproteinases can modulate the biological activity of sFasL by releasing the stalk region.

2. To identify the mechanisms that modify the structure and the activity of human sFasL in the airspaces of patients with ARDS:
   a. To determine whether neutrophil-derived oxidants facilitate the aggregation and modify the biological activity of sFasL, and
   b. To determine the molecular mechanisms and the key amino acids involved in the structural and functional modifications of sFasL under oxidative conditions.

6. – MATERIAL AND METHODS

6.1. REAGENTS

Commercial variants of recombinant human and mouse sFasL

Three commercial forms of recombinant human sFasL (rh-sFasL) were used in this project. The rh-sFasL from Axxora-Alexis Co. (Alexis SuperFasL, Cat# 522-020-3005) was used to develop the animal model of human sFasL-mediated lung injury in mouse lungs. Another form of rh-sFasL from Peprotech Inc. (Cat# 310-03) was used in *in vitro* models of rh-sFasL incubated in ARDS BAL fluid, or exposed to oxidative and reducing conditions. These two rh-sFasL proteins corresponded to the whole extracellular domain of FasL. In addition to these proteins, a short form of rh-sFasL from R&D Systems (Cat# 126-FL/CF) was used in *in vitro* experiments to determine the level of aggregation of all variants of human sFasL. A short form of a commercial recombinant mouse sFasL from R&D Systems (Cat# 526-SAC/CF) was also used to study the effect of oxidation in multimerization of sFasL.

Mice: C57BL/6 (wild-type) and MRL/MpJ-Faslpr/2J (lpr Fas-deficient mice) with a C57BL/6 background were obtained from Jackson Laboratories (Bar Harbor, ME).

Cultured cells: Jurkat cells (human T cell lymphoblast-like cell, clone E6-1) were purchased from ATCC. HEK293 cells (Human Embryonic kidney cells, FreeStyle 293 R97007) were purchased from Invitrogen.

Antibodies (Ab) for human proteins:

For western-blotting of human FasL: Goat anti-human FasL polyclonal Ab (Peprotech Inc., Cat# 500-P184G), HRP conjugated rabbit anti-goat Ab (KPL, Cat# 141306), mouse anti-human FasL monoclonal Ab (BD Biosciences, Cat# 556387), HRP conjugated AffiniPure F(ab')2 fragment donkey anti-mouse Ab (Jackson ImmunoResearch Cat# 715-036-151).

For ELISA: Human sFas ligand ELISA Kit (Cat#5255 MBL Nagoya Japan).

For FasL purification: mouse anti-human FasL monoclonal Ab (R&D Systems, MAB126, 0.5 ml/vial).

Antibodies (Ab) for mouse proteins:

For western-blotting: Rat anti-mouse FasL Ab (R&D Systems, Cat# MAB5262), HRP conjugated goat anti-rat Ab (Amersham, Cat# NA 935V).

For ELISA: Mouse Fas Ligand DuoSet ELISA (R&D Systems, Cat# DY526).

For FasL purification: Rat anti-mouse FasL Ab (R&D Systems, Cat# MAB5262).
For immunohistochemistry of caspase-3: Rabbit anti-cleaved caspase-3 polyclonal Ab (Cell Signaling, Cat# 9661S), goat anti-rabbit biotinylated Ab (Zymed®, Cat# 65-6140).

Enzymes: Myeloperoxidase (MPO-Sigma-Aldrich, Cat# M6908), Catalase (Sigma-Aldrich, Cat# C-3515), Human metalloproteinase-7 (MMP-7). Calbiochem, Cat# 444270)

Other reagents: L-methionine (Sigma-Aldrich, Cat# M5308), hypochlorous acid (HOCl-Fisher Scientific, Cat# SS290-1), hydrogen peroxidase (H_2O_2) (Sigma-Aldrich, Cat# 095K3774), dimethyl sulfide (Sigma-Aldrich, Cat# 41624), hydrochloric acid (J.T. Baker Inc., Cat# 9535-33), iodoacetamide (IAM) (Sigma-Aldrich, Cat# I1149), phosphate buffer saline (PBS 10X) (Invitrogen Corporation). Tween-20 (Sigma-Aldrich), bovine serum albumin (BSA) (Spectrum Chemical), peroxi-Block (Zymed, Cat# 50-2015), Alamar Blue (BioSource, Cat# AL-01-100), Dako Serum-Free Protein Block (Dako, Cat# X0909), Avidin-Biotin Complex-HP (ABC-HP) (VECTOR Labs., Cat# PK-6101), diaminobenzidine substrate (Zymed, Cat# 00-2014) and methyl green (Trevigen, Cat# 4800-30-18). The rest of the reagents used in this project were purchased from Sigma-Aldrich.

6.2. EXPRESSION OF HUMAN AND MOUSE sFASL

6.2.1. CLONING OF sFASL

Human and mouse of sFasL cDNA sequences were amplified by using a standard PCR technique with DNA polymerase (Easy-A High-Fidelity PCR Cloning Enzyme Cat#600400 Strategene). The sequences of PCR primers used for amplification of human and mouse cDNA were as follows:

Cloning Human sFasL:

Short human sFasL:
5'-AAGCAAATAGGCCACCCCAG-3' Forward
5'-AGTTTCACCGATGGCTCAGG-3' Backward

Long human sFasL:
5'-CAGCTCTTCCACCTACAGAAGGAGC-3'
5'-GTCCCCAAAACATCTCTCTTG-3' Backward

Cloning Mouse sFasL:

Short mouse sFasL:
5'-CCCAGTACACCCTCTGAAAAAAAAGAGCCG-3' Forward
5'-GGGGGCTCATGATGCAGGCATTAAGGACC-3' Backward
Long mouse sFasL:
5'-CAGCTCTTCCACCTGCAG-3' Forward
5'-AGACAATATTCCTGGTGCCC-3' Backward

The PCR conditions were: 95°C, 2 min, then 95°C, 40 sec, 55°C, 30 sec, 72°C, 1 min, for 35 cycles, and 72°C, 7 min. The PCR products were visualized in agarose gels containing ethidium bromide, excised from the gel and then extracted from the gel using Amicon Ultrafree-DA (Cat# 42600 Millipore). The purified cDNA was ligated into pSecTag/FRT/V5-His-TOPO vector in pSecTag/FRT/V5-His-TOPO Expression Kit (Cat# K6025-01 Invitrogen) according to the manufacturer's instructions.

For cloning reaction, the recombinant vectors containing human or mouse sFasL cDNA were transformed by heat shock into competent TOP10 *E. coli*. The transformed bacteria were plated on LB agar plates containing ampicillin. The optimal cell transformation was screened by PCR amplification. Then, the sequences of the cloned pSecTag/cDNA-sFasL plasmids were confirmed by BigDye Terminator v3.1 Cycle Sequencing Kit (Cat# 4337454 Applied Biosystems).
The large scaled endotoxin-free plasmids were isolated from bacteria by using EndoFree Plasmid Maxi Kit (Cat#040506 Qiagen Inc.) for the transfection.

6.2.2. SITE-DIRECTED MUTAGENESIS

The wild-type mouse sFasL cDNA was mutated by site-directed mutagenesis in order to generate a mutant mouse sFasL encoding the same number of methionine residues and in the same location as those in human sFasL. Point-mutations in a total of 5 codons in the plasmid encoding wild-type mouse sFasL cDNA were performed by

using QuikChange II site-directed mutagenesis kit (Cat# 200523, Stratagene) and the following mutagenic primers:

91 codon:
5'-GTATCCTGAGGATCTGGTGATGATGGAGGAGAAGATGATG-3' Forward
5'-CATCATCTTCTCCTCCATCATCACCAGATCCTCAGGATAC-3' Backward
105 codon:
5'-CTGCACTACTGGACAGATGTGGGCCCACAGCAGCCACCTG-3' Forward
5'-CAGGTGGCTGCTGTGGGCCCACATCTGTCCAGTAGTGCAG-3' Backward
96 and 97 codons:
5'-GCTAATGGAGGAGAAGATGATGAACTACTGCACTACTGG-3' Forward
5'-CCAGTAGTGCAGTAGTTCATCATCTTCTCCTCCATTAGC-3' Backward
25 codon:
5'-GAACCCCCACTCAAGGTCCATGCCTCTGGAATGGGAAGAC-3' Forward
5'-GTCTTCCCATTCCAGAGGCATGGACCTTGAGTGGGGGTTC-3' Backward

The plasmids containing the WT or the Mutant sFasL cDNA were transformed into XL1-Blue supercompetent cells (E. coli dam$^+$, Stratagene) by heat shock. The transformed cells were spread on LB-agar plates containing 100 μg/mL ampicillin. To verify the transformation efficiency, some cultured cell colonies were directly inoculated into a PCR cocktail for DNA amplification and visualized by agarose gel electrophoresis. The plasmids were isolated from the same verified colonies with Wizard Plus SV Minipreps DNA Purification System (Promega, Cat# A1330). The isolated plasmids were processed for sequencing using BigDye Terminator v3.1 Cycle Sequencing Kit to verify that the selected colonies contained the desired mutations.

6.2.3. DNA TRANSFECTION INTO MAMMALIAN CELLS

Human Embryonic kidney (HEK) cells were cultured in FreeStyle 293 expression medium (Gibco) without serum in a humidified 8% CO_2 incubator at 37ºC, while shaking. The plasmids pSecTag/cDNA-sFasL coding the mouse or human sFasL (250 mg) were incorporated to liposomes (293fectin, Invitrogen) in Opti-MEM I (Invitrogen) for 30 min at room temparature. HEK cells ($1x10^6$ cells/mL) were transiently transfected using the liposomes containing the plasmid pSecTag/cDNA-sFasL

according to the manufacturer's instructions. The cell supernatant was harvested 3 days or 6 days after cell transfection; then filtered and stored at –20ºC.

6.2.4. PROTEIN PURIFICATION BY IMMUNOAFFINITY COLUMN

6.2.4.1. Preparation of immunoaffinity column

Mouse monoclonal antibody to human Fas Ligand (R&D Systems, Cat# MAB126) and rat anti-mouse Fas Ligand antibody (R&D System) at the concentration of 500 μg/mL were first dialyzed overnight in a slide-A-Lyzer (Pierce, Cat# 66425) against 1000 mL of coupling buffer at 4°C while stirring. The column (Hi Trap NHS-activated HP 1 ml column Cat# 17-0716-01 GE Healthcare Piscataway NJ) was washed with pre-chilled 1 mM HCl (10 mL x 3 times at 5 mL/min). Then, the dialyzed anti-FasL antibody was injected into the column at 5 mL/min. The column was then left standing for 30 min at room temperature, following by three series of washes using buffer A and B alternatively and according to manufacture's instructions. Dulbecco's PBS was used for the last wash and the column was stored at 4°C. The compositions of coupling buffer and wash A and B buffers are described below:

– Coupling buffer**:** 0.2 M $NaHCO_3$ and 0.5 M NaCl, pH 8.3
– Buffer A**:** 0.5 M Ethanolamine and 0.5 M NaCl, pH 8.3
– Buffer B: 0.1 M Sodium Acetate and 0.5 M NaCl, pH 4.0

6.2.4.2. Protein purification by immunoaffinity column

The immunoaffinity column was washed first with an elution buffer (0.1 M glycine and 1M NaCl buffer pH2.5) for 10 min followed by PBS using the Pharmacia Peristaltic Pump. The supernatant collected from the transfected HEK123 cells was filterred through a 0.45 μm pore filter. The transfected HEK cell supernatant containing the variants of human or mouse sFasL was applied through the column and the flow through was collected. The column was thoroughly washed with PBS. The protein bound to the column was eluted with the elution buffer and collected in separate fraction tubes containing 100 μl of 1 M phosphate buffer pH 7.4. After washing the column again with PBS, it was filled with 5 mL of 0.02% NaN_3 PBS and stored at 4ºC. The concentrations of human and mouse variants of sFasL protein were determined by ELISA (Cat# 5255 MBL Nagiya Japan or R&D Systems) in each fraction. Fractions

with the highest concentrations of sFasL were pooled and the final concentrations of sFasL were determined again by ELISA. The purified proteins in solution were stored at –20ºC.

6.2.4.3. Dialysis of purified sFasL proteins

One of the purposes of this study was to explore the level of multimerization of sFasL proteins under oxidative conditions. We observed that the presence of glycine in the purified sFasL elution prevented protein oxidation. In order to reduce the content of glycine, the sFasL protein eluated were dialyzed several times in dialysis cassettes (Slide-A-Lyzer, 10,000 MWCO, Pierce) against PBS 1X, pH 7.4 at 4ºC.

6.3. CONCENTRATIONS OF sFASL MEASURED BY ELISA

6.3.1. ELISA OF HUMAN sFASL

Human sFasL was measured by the MBL ELISA kit (cat# 5255), which includes microwells coated with anti-human FasL antibody. The samples and the standards were diluted 1:2 with assay diluent, and added to the antibody coated microwells. After 60 min of incubation, the well contents were discarded and the wells were washed several times. Next, the wells were incubated with conjugated solution containing the peroxidase conjugated anti-human FasL monoclonal antibody for 60 min. After several washes, the wells were incubated with substrate solution for 30 min. Then, the reaction was stopped and the absorbance of each well was read at 450 nm. All the steps were performed at room temperature. The low detection limit of human FasL ELISA was 75 pg/mL.

6.3.2. ELISA OF MOUSE sFASL

Mouse sFasL was measured by the R&D Systems ELISA kit (cat# DY526). A 96-well plate was coated with the corresponding capture anti-mouse FasL antibody overnight at room temperature. The capture antibody was aspirated and the wells were washed three times with 0.05% Tween-20 in PBS. The wells were blocked with 1% BSA diluted in PBS for 1 hour, and washed three times. Samples containing mouse, human

sFasL or standards were added and incubated for 2 hours followed by another aspiration/wash step. Next, the corresponding detection anti-mouse FasL biotinylated antibody was applied to the wells for 2 hours. The wells were washed three times and then incubated with streptavidin-HRP solution for 20 min, washed three more times and developed with TMB substrate solution for another 20 min on the dark. The reaction was stopped by the addition of 1M phosphoric acid. The optical density was determined immediately following this procedure by using a microplate reader set to 450 nm. All of the procedures were performed at room temperature. The low detection limit of mouse FasL ELISA was 15 pg/mL.

6.4. EXPERIMENTAL PROTOCOL IN ANIMAL MODELS

6.4.1. ANIMAL PROTOCOL

The animal protocols were approved by the Animal Care Committee of the Veterans Administration Puget Sound Health Care System (Seattle, WA). Male mice weighing 25–30 g were anesthetized with inhaled 2-5% isoflurane, and then placed on an inclined surface. The larynx was visualized and intubated with a gavage tube attached to a 1.0-mL syringe containing 100 μl of water. Intubation of the trachea was verified by movement of the water bubble with the animal's respiratory efforts. After endotracheal intubation was confirmed, one single dose of 25 ng/g of human sFasL (LPS-free) was instilled into the lung. After instillation, the gavage tube was removed, and the mice were returned to their cages, allowed to recover from anesthesia, and provided with free access to food and water. The mice were monitored throughout the study. Our time-points, depending on the experiments, were 6 hours and 16 hours after intratracheal instillation.

The mice were euthanised at 6 or 16 hours post-instillatiton or at any time if they developed weight loss >15% of the original body weight, had evidence of respiratory distress, or at least three of the following: 1) dehydration (evaluated by skin tenting), 2) lethargy and decreased movement, 3) pale eyes, 4) loose stools, 5) nasal and/or ocular discharge, or 6) neuromuscular signs (uncoordination or seizures).

6.4.2. SAMPLING

Sixteen hours after intratracheal instillation, the mice were killed with an intraperitoneal injection of pentobarbital (120 mg/kg) and exsanguinated by closed intracardiac puncture at 16 hours post-instillation. The thorax was opened and the trachea cannulated and secured.

Snap freezing of left lung: The left hilum was clamped and sutured with 2-0 silk suture, then, the left lung was removed and flash frozen in liquid nitrogen.

Bronchoalveolar lavage (BAL): After removing the left lung, bronchoalveolar lavage (BAL) was performed in the right lung by instillation of 0.9% NaCl containing 0.6 mmol/L ethylenediaminetetraacetic acid (EDTA) in four separate 0.5 mL aliquots. The BAL fluid was recovered by gentle suction and placed on ice for immediate processing.

Fixation: Immediately after the BAL procedure, the right lung was fixed by inflation with 4% paraformaldehyde at a transpulmonary pressure of 15 cmH_2O, and immersed into the same fixative overnight at 4ºC. Next day, lungs were processed for paraffin-embedding.

6.4.3. EXPERIMENTAL DESIGN

6.4.3.1. Dose-response of human sFasL in the lungs of mice

We developed a preliminary experiment to determine the most efficient dose of recombinant human sFasL to induce lung injury in mice at an early time-point. Male C57BL/6 mice were challenged with one intratracheal instillation of recombinant human sFasL (rh-sFasL) at different doses diluted in sterile phosphate-buffer: 0.3 ng/g (n= 5), 1 ng/g (n=5), 3 ng/g (n= 8), 8.3 ng/g (n=4), 25 ng/g (n=4). The mice were euthanized at 6 hours post-instillation. The administration of rh-sFasL at the dose of 25 ng/g induced the maximal response in PMN recruitment in the lungs.

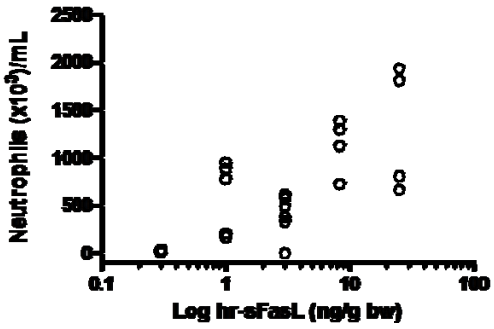


Figure 6.1. PMN recruitment in mouse lung treated with different concentrations of human sFasL. Mice received one intratracheal instillation of different doses of recombinant human sFasL (SuperFasL, Alexis Co.): 0.3 ng/g (n= 5), 1 ng/g (n=5), 3 ng/g (n= 8), 8.3 ng/g (n=4), 25 ng/g (n=4). The number of PMN was determined in the BAL fluid collected from these mice at 6 hours after intratracheal instillation. Results shown in scattered dots. Each dot represents one mouse. X axis represents the doses expressed in $\log_{10}$ numbers.

6.4.3.2. Human sFasL induces lung injury through Fas activation in mice

We first developed a mouse experiment to determine whether human sFasL is cytotoxic in the lungs of mice through Fas activation. Two strains of mice were used: male C57BL/6, which express Fas (wild-type mice), and *lpr* mice, which are natural mutants deficient in Fas on a C57BL/6 background (MRL/MpJ-Faslpr/2J). All the mice were obtained from the Jackson Laboratories (Bar Harbor, ME). We studied the following groups:

I. Group control Normal: C57BL/6 mice expressing Fas (wild-type mice) received no intratracheal instillation (n=5)
II. Group control PBS: C57BL/6 mice expressing Fas (wild-type mice) received one intratracheal instillation of PBS only. (n=12)
III. Group WT-human sFasL: C57BL/6 mice expressing Fas (wild-type mice) received one intratracheal instillation of human sFasL (25 ng/g) (n=10)
IV. Group of *lpr*-human sFasL: *lpr* mice deficient in Fas received one intratracheal instillation of human sFasL (25 ng/g) (n=10)

All the mice were euthanized 16 hours after intratracheal instillation. The degree of lung injury was evaluated by the presence of: 1) structural alterations of the alveolar wall, 2) cell death, and 3) alveolar-capillary barrier dysfunction.

6.4.3.3. Comparison of the activity of variants of human sFasL in mice

Male C57BL/6 mice expressing Fas were used to compare the activity of two variants of human sFasL with different N-terminal (Long and Short human sFasL) in mouse lungs *in vivo*. These mice were obtained from Jackson Laboratories (Bar Harbor, ME). We studied the following groups:

I. Group control Normal: C57BL/6 mice expressing Fas received no intratracheal instillation (n=3)
II. Group control PBS: C57BL/6 mice expressing Fas received one intratracheal instillation of PBS only. (n=7)
I. Group Human Long-sFasL: C57BL/6 mice expressing Fas received one intratracheal instillation of human Long-sFasL (25 ng/g) (n=5)
II. Group of Human Short-sFasL: C57BL/6 mice expressing Fas received one intratracheal instillation of human Short-sFasL (25 ng/g) (n=5)

All the mice were euthanized 16 hours after intratracheal instillation. The degree of lung injury was evaluated by the presence of: 1) structural alterations of the alveolar wall, 2) cell death, and 3) alveolar-capillary barrier dysfunction.

6.5. HISTOLOGICAL METHODS AND IMMUNOHISTOCHEMISTRY

6.5.1 HEMATOXILIN AND EOSIN STAINING OF MURINE LUNG

Lung sections were stained with hematoxylin and eosin for light microscopy. Multiple tissue sections derived from C57BL/6 wild-type or *lpr* mice were deparaffinized in xylene for 20 min, then rehydrated in graded ethanol solutions to PBS. Staining was performed with Harris hematoxylin, then eosin Y. The sections were rinsed with H2O, dehydrated in graded ethanol solutions, immersed in xylene, and mounted in Permount.

6.5.2. CASPASE-3 IMMUNOHISTOCHEMISTRY

The sections were deparaffinized by heating at 57°C for 60 min and rehydrated by washing twice in xylene for 5 min, twice in 100% ethanol for 3 min, twice in 95% ethanol for 3 min, and once in deionized H_2O for 5 min. The slides were then washed three times in PBS for 5 min and treated with 0.3% Triton X-100 for 30 min at room temperature. After washing in PBS three times for 5 min, endogenous peroxidases were blocked with Peroxo-Block (Zymed) for 2 min at room temperature. The slides were rinsed twice with PBS for 5 min, and the samples were treated with citrate buffer (Vector Laboratories) in a microwave for 15 min at medium setting. The slides were cooled to room temperature for 10 min, rinsed twice with PBS for 5 min, and blocked with Dako Serum-Free Protein Block (Dako, Catalog # X0909) for 30 min at room temperature. The tissues were then labeled with rabbit anti-cleaved caspase-3 polyclonal Ab (Cell Signaling) overnight at 4°C in a moist chamber. After washing in PBS three times, the tissues were labeled with biotinylated goat anti-rabbit biotinylated Ab (Zymed) for 40 min at room temperature and washed in PBS three times. The slides were then labeled with avidin-biotin complex-HP (ABC-HP, VECTOR Labs) for 30 min at room temperature, rinsed three times with PBS, and developed in a moist chamber with diaminobenzidine substrate (Zymed) for 10 min. The slides were rinsed with running deionized H_2O for 5 min, counterstained with 1% methyl green (Trevigen) for 6 min. The slides were dehydrated with ethanol, incubated in xylene for 5 min, and mounted with Permount.

6.6. MEASUREMENTS IN MOUSE LUNG TISSUE

6.6.1. LUNG TISSUE HOMOGENIZATION

The left lung was weighed and then homogenized in 1.0 mL of sterile distilled H_2O using a hand-held homogenizer. Each lung homogenate was divided into aliquots for cytokine, caspase-3 activity and myeloperoxidase (MPO) measurements. For cytokine and caspase-3 activity measurements, the homogenate aliquot was mixed with a lysis buffer containing 0.5% Triton X-100, 150 mM NaCl, 15 mM Tris, 1 mM $CaCl_2$, and 1 mM $MgCl_2$ (pH 7.40), incubated for 30 min at 4ºC, and then centrifuged at 10,000 x *g* for 20 min. The supernatants were stored at -80 ºC. For MPO measurements, the

homogenate aliquot was vigorously mixed with 50 mM potassium phosphate (pH6.0) with 5% hexadecyltrimethyl ammonium bromide (Sigma-Aldrich) and 5 mM EDTA. The mixture was sonicated and spun at 12,000 x *g* for 15 min at 25 ºC, and the supernatant was stored at -80 ºC.

6.6.2. CYTOKINE EXPRESSION BY ELISA

The cytokines IL-1β, IL-4, IL-6, IL-10, IL-12, KC, MIP-2, MCP-1, TNF-α and IFN-δ were measured in lung homogenates using Fluorokine MultiAnalyte Profiling kits (R&D Systems.) for a multiplex fluorescent bead assay (Luminex, Austin, TX). After dilution of the samples, the sensitivities of these immunoassays were 37,9 pg/mL for IL-1β, 11.25 pg/mL for IL-4, 10,53 pg/mL for IL-6, 7.47 pg/mL for IL-10, 23,83 pg/mL for IL-12, 13.08 pg/mL for KC, 6.69 pg/mL for MIP-2 , 54.15 pg/mL for MCP-1, 6.82 pg/mL for TNF-α and 22.19 pg/mL for INF-δ.

6.6.3. ENZYMATIC ASSAYS

6.6.3.1. Myeloperoxidase activity

Neutrophil infiltration of the lung was evaluated by measuring myeloperoxidase (MPO) activity in the sypernatant of lung homogenates. Myeloperoxidase activity was measured in lung homogenates using the Amplex Red fluorometric assay, following instructions from the manufacturer (Molecular Probes, Eugene, OR). The supernatant was mixed 1:15 with assay buffer and read at 490 nm. MPO units were calculated as the change in absorbance over time. The assay buffer consisted of 100 mM potassium phosphate, pH 6.0, 0.083 mL H_20_2 (Sigma; 30% stock diluted 1:100), and 0.834 mL o-dianisidine hydrochloride (Sigma; 10 mg/mL). MPO activity was calculated as the change in absorbance over time measured by an espectrophotometer, as described previously.

6.6.3.2. Caspase-3 activity

Caspase-3 activity in lung homogenates was measured with the caspase-3/CPP32 Fluorometric Assay kit (Biovision, Mountain View, CA). Fifty-microliter aliquots of lung homogenate were diluted 1:2 in assay reaction buffer containing 10 mM DTT, and

incubated for 2 hours at 37°C with the caspase-3-specific substrate DEDV-AFC (50μM). Active caspase-3 cleaves a variety of molecules that contain the amino acid motif DEVD. The cleavage of this peptide releases AFC (7-amino-4-trifluoromethyl coumarin) that, when excitated by light at 400 nm, emits fluorescence at 505 nm. Caspase-3 activity is directly proportional to the fluorescence signal (505 nm) measured using a fluorescent microplate reader.

6.7. MEASUREMENTS IN MOUSE BRONCHOALVEOLAR LAVAGE (BAL) FLUID

6.7.1. TOTAL CELL AND DIFFERENTIAL COUNTS IN MOUSE BAL FLUID

The bronchoalveolar (BAL) fluid aliquots from each mouse were pooled and processed immediately for total cell counts and differentials. Total cell counts were performed with a hemocytometer, whereas differential counts (cytology) were performed on cytospin preparations stained with the Diff-quick method (Andwin Scientific, Addison, IL), and a minimum of 200 cells were counted.The remainder of the lavage fluid was spun at 200 x*g* for 30 minutes and the supernatant was removed aseptically and stored in individual aliquots at – 80ºC.

6.7.2. PERMEABILITY MEASUREMENTS

The total protein concentration in BAL fluid was measured using the bicinchoninic acid method (BCA assay; Pierce, Rockford, IL). The concentration of IgM in BAL Fluid was measured with a specific mouse immunoassay (R&D Systems). After dilution of the samples, the lower limit of detection of the IgM assay was 20 ng/mL.

6.8. CELL CULTURE EXPERIMENTS IN JURKAT CELLS

6.8.1. INCUBATION OF JURKAT CELLS

Human Jurkat cells T Lymphocytes (ATCC) were grown in RPMI-1640 medium supplemented with 10% fetal bovine serum at 37ºC, 5% CO2. For the bioassay

experiments, Jurkat cells were seeded in Costar 96-well tissue culture plates (Corning Inc.) at $2x10^5$ cells/well in serum-free media supplemented at a 50% concentration with serum-free media containing serial concentrations of samples containing sFasL or media only. After incubation for 18 hours at 37ºC, 5% CO2, cell survival was assessed using Alamar Blue assay.

6.8.2. CYTOTOXICITY MEASURED BY ALAMAR BLUE ASSAY

Cell viability was assessed with Alamar Blue assay (BioSource International, Camarillo CA) which incorporates a fluorometric/colourimetric growth indicator based on detection of metabolic activity. This is an oxidative-reduction (redox) indicator that both fluoresces and color changes in response to reducing equivalents generated during cellular metabolism and cell growth. Basically, the oxidized form of the redox indicator (non-fluorescent, blue) change to a reduced form (fluorescent, red) in the presence of live cells. Alamar Blue reduction results in a shift in light fluorescence from 590 nm to 530 nm.

After incubation for 16 hours with conditional media, 10% of Alamar Blue was added to the wells. The cells were then incubated for 4 hours at 37ºC in 5% CO2. After incubation, fluorescence was measured at 530-nm excitation and 590-nm emission using a CytoFlour II fluorometer (PerSeptive Biosystems, Framingham, MA). Data are shown as percent of survival or as percent of cell death.
Percent of survival was calculated as follows:
Survival (%) = (experimental fluorescence x 100)/live cell fluorescence.
Cell death (%) = [(live cell fluorescence-experimental fluorescence)/live cell fluorescence] x100.
Live cell fluorescence corresponds to fluorescence of cells in media only.

6.9. INCUBATION OF sFASL IN HUMAN BAL FLUID

Exogenous recombinant human sFasL (rh-sFasL) was added to PBS or pooled BAL fluid samples from normal volunteers, patients at-risk or with ARDS, and was incubated for 90 minutes at 37ºC. At the end of the incubation, the HOCl scavenger L-methionine was added to the samples. When indicated, rh-sFasL was incorporated to the ARDS BAL fluid after the addition of L-methionine to prevent protein oxidation. As control for

Western-blot analysis, the same volume of each BAL fluid samples without exogenous human sFasL protein was also incubated. As indicated in each experiment, the samples (with and without exogenous sFasL) were or were not boiled for 5 min and subjected to electrophoresis without reducing agents in SDS-PAGE gels.

6.10. OXIDATION OF sFASL

6.10.1. OXIDATION OF sFASL BY MIELOPEROXIDASE-H_2O_2-Cl^- SYSTEM

Commercial recombinant human and mouse sFasL proteins (0.06 μM) were incubated in 25 μL of PBS-DTPA in the presence of 100 mM NaCl and serial concentrations of myeloperoxidase (10, 25 and 50 nM) and H_20_2 (2, 5, 10, and 20 μM) for 60 min at 37ºC. Then, the molecular weight of sFasL subspecies was determined by SDS-electrophoresis, with or without reducing sample buffer as specified in each experiment, and Western-blotting.

6.10.2. OXIDATION OF sFASL BY HYPOCHLOROUS ACID (HOCl)

Recombinant human and mouse sFasL, including the short wild-type form of mouse sFasL (WT sFasL) and the short methionine-mutated mouse sFasL (Met-sFasL) proteins expressed in our laboratory, were treated with serial dilutions of HOCl from 0.06 to 30 μM for 30 min at 37ºC. The reaction was stopped by adding 2.5 mM of free L-methionine. When indicated, sFasL protein was added to the oxidising buffer once the oxidative reaction was terminated with free L-methionine to prevent sFasL from oxidation. These non-oxidized samples were used as controls. The molecular weight of sFasL subspecies was determined by SDS-electrophoresis, with or without reducing sample buffer as specified in each experiment, and Western-blotting.

6.11. REDUCTION OF sFASL

6.11.1. REDUCTION OF sFASL BY DITHIOTHREITOL (DTT)

In order to determine whether disulfide bonds are involved in oxidation-mediated multimerization of human sFasL, we incubated a mildly oxidized recombinant human sFasL protein with the reducing agent dithiothreito (DTT) followed by iodoacetamide. Iodoacetamide is an alkylating sulfhydryl reagent commonly used to bind covalently with cysteine, so the protein cannot form disulfide bonds. Briefly, a recombinant human sFasL (rh-sFasL, Peprotech Inc.) at the concentration of 10,000 ng/mL was incubated in 50 μL 1X PBS containing 5 mM of the reducing agent dithiothreitol (DTT) at 37ºC. After 1 hour, 10 mM of iodoacetamide (IAM) was added to the reaction and was incubated at room temperature for another 30 min. As control, rh-sFasL was diluted in 1X PBS without either DTT or IAM. To stop the reaction and to reduce the content of DTT and IAM, the samples were diluted 1:5 in 1X PBS, and then filtered in albumin pre-coated centrifugal filter columns with a 3,000-kDa molecular weight cut-off (Microcron Ultracel YM-3, Millipore) by centrifugation at 10,000 x *g* for 25 min at 4ºC. The molecular weight of sFasL subspecies was determined by SDS-electrophoresis, without reducing sample buffer, and Western-blotting.

6.11.2. REDUCTION OF sFASL BY DIMETHYL SULFIDE (Me_2S)

In order to determine whether oxidation of methionine residues is involved in oxidation-mediated multimerization of human sFasL, we incubated a mildly oxidized recombinant human sFasL with dimethyl sulfide. Dimethyl sulfide (Me_2S) specifically reduces the methionine sulfoxide (oxidized methionine) back to methionine sulfide (non-oxidized methionine) as described by Y. Shechter et al. [630]. Briefly, a recombinant human sFasL (rh-sFasL, Peprotech Inc.) was reduced by dissolving 400 ng of rh-sFasL in 100 μL of 0.1 M Me_2S and 10 M HCl. The reaction was allowed to proceed at room temperature for reaction times ranging from 10 min to 2 hours. To stop the reaction and reduce the content of Me_2S and HCl, the samples were diluted 1:30 in 10X PBS, pH 7,4, and then filtered in albumin pre-coated centrifugal filter columns with a 3,000-kDa molecular weight cut-off (Microcron Ultracel YM-3, Millipore) by centrifugation at 14.000 x *g* for 25 min at 4ºC. After filtration, the samples were diluted again in 50 μL of ddH_2O. The

molecular weight of sFasL subspecies was determined by SDS-electrophoresis, without reducing sample buffer, and Western-blotting.

6.12. MOLECULAR WEIGHT ANALYSIS OF sFASL

6.12.1. ELECTROPHORESIS AND WESTERN-BLOTTING OF EXOGENOUS sFASL

The molecular mass and the level of aggregation of sFasL proteins were determined under different conditions by electrophoresis in both SDS and Native gel:

6.12.1.1. Electrophoresis in SDS gel

Sodium Dodecyl Sulfate Polyacrylamide polyacrylamide gel electrophoresis (SDS PAGE): In the SDS-PAGE, the charge shift molecule is Sodium Dodecyl Sulfate Polyacrylamide (SDS). The SDS denatures proteins and binds to proteins conferring a net negative charge allowing the proteins to migrate towards the anode. The SDS makes the proteins lose their quaternary structure and converts the oligomeric proteins into their constituent polypeptides. Therefore, in SDS-PAGE the electrophoretic mobility of proteins depends primarily on their molecular mass.

Samples of the BAL fluid or the oxidative or reducing reactions containing exogenous recombinant human or mouse sFasL were added to 2X sample Laemmli buffer without reducing agents. Samples were either not boiled or boiled for 5 min as mentioned in each experiment. The amount of human and mouse sFasL proteins loaded per well in the 4-20%-SDS gel (Lonza) were 10 ng and 2 ng, respectively.

6.12.1.2. Electrophoresis in native gel

Native polyacrylamide gel electrophoresis (Native PAGE): The Native PAGE gel system is based on the Blue Native Polyacrylamide gel electrophoresis technique developed by Schagger and von Jagow in 1991 that uses Coomassie G-250 as a charge-shift molecule [631]. In Native-PAGE, the coomassie G-250 binds to proteins and confers a net negative charge. In contrast to SDS, G-250 charges the proteins while maintaining their native state without any protein denaturation. The G-250 is present in

the cathode buffer to provide a continuous flow of G-250 into the gel, and is added to samples containing non-ionic detergent prior to loading the samples onto the gel. The gels do not contain any G-250. "Native" or "non-denaturing" gel electrophoresis is run in the absence of SDS. While in SDS-PAGE the electrophoretic mobility of proteins depends primarily on their molecular mass, in native PAGE the mobility depends on the protein's charge, the folded conformation and its hydrodynamic size. Thus, larger structures like multimers have lower mobility though the gel than monomers of a particular protein. Therefore, we used Native PAGE to study the structural conformation and aggregation (self-association) of sFasL.

The samples are mixed with 4x native PAGE sample buffer and 5% G-250 sample additive (Invitrogen) without any reducing agent. This mixture is then added to a pre-cast 4-16% Bis-Tris polyacrylamide gel (NativePAGE Novex® gel Cat# BN1004BOX Invitrogen) that operates near to neutral pH. The proteins are run in the gel using a NativePAGE running buffer with a cathode additive for the upper buffer chamber and without the cathode additive for the lower buffer chamber according to manufacturer's instructions (NativePAGE™ Novex® Bis-Tris gel system, Cat# K6025-01, Invitrogen)

6.12.1.3. Western-blotting

After electrophoresis in SDS gel, the proteins were transferred to nitrocellulose membrane (Hybond-ECL NC) with Bjerrum-Schaffer Nielsen transfer buffer at 100 vol at 4ºC or room temperature as specified in each experiment. After electrophoresis in native gel, the proteins were transferred to a PVDF membrane with NuPAGE transfer buffer (Cat# NP0006-1, Invitrogen) at 100 vol at room temperature. The membrane was blocked first with 0.2% SuperBlock (Pierce) buffer and 0.05% Tween-20 at room temperature for 1 hour and discarded afterwards. Then the membrane was incubated overnight at 4ºC with the corresponding primary antibodies diluted in 0.2% SuperBlock/0.05% Tween-20 buffer (see below). After thoroughly washing with 1 x PBS 0.05% Tween-20, the membrane was incubated for one hour at room temperature with HRP conjugated secondary antibody also diluted in 0.2% SuperBlock/0.05% Tween-20 buffer (see below). After 3 washes in wash buffer, peroxidase activity was detected by chemiluminescence using Supersignal West Femto Maximum Sensitivity Substrate (Super Signal West Femto, Cat# 34094 Pierce).

1. **For human sFasL**:
   - Primary antibody: Mouse anti-human FasL antibody (BD Biosciences) diluted 1:250 or Goat anti-human FasL antibody (Peprotech) diluted 1:500

   - Secondary antibody: HRP-Donkey anti-mouse IgG (Jackson ImmunoResearch) diluted 1:1x10^5 or HRP-Rabbit anti-goat IgG (KPL) diluted 1:1x10^5

2. **For mouse sFasL detection:**
   - Primary antibody: Rat anti-mouse FasL antibody (R&D System) diluted 1:250
   - Secondary antibody: HRP-Goat anti-rat antibody (Amersham) diluted 1:10000

6.12.2. ELECTROPHORESIS AND WESTERN-BLOTTING OF ENDOGENOUS HUMAN sFASL IN BAL FLUID

Samples of BAL fluid from four normal volunteers, four patients at-risk for ARDS and four patients with established ARDS (day 3) were selected from our BAL fluid bank. The samples were spun at 18,000 x *g* for 10 min at 4ºC. In previous experiments, we noticed that human albumin and IgG present in BAL fluids caused nonspecific background in Western blotting analysis of sFasL. In order to remove human albumin and IgG, 300 μl of each BAL fluid was incubated with anti-human albumin and anti-human IgG antibodies coupling agarose beads (ProteoSeek Antibody-Based Albumin/IgG Removal Kit, Cat# 89876, Pierce, Rockford IL) for 2 hours at room temperature. After incubation with the beads, BAL fluid was separated from the beads by filtration, and albumin/IgG depleted BAL fluid was concentrated 10-fold by spinning at 5,000 x *g* for 10 min at 4ºC using a ultrafiltration device with a 5,000-kDa molecular weight cut-off (Ultrafree-MC Centrifugal Filter Units Millipore). Next, each sample was mixed with an equal volume of 2x Laemmli SDS-PAGE sample buffer and separated in 4-12% SDS-PAGE under non-reducing conditions. The endogenous sFasL was detected by Western blotting as described above using biotinylated goat anti human sFasL IgG or biotinylated goat isotype control IgG with streptavidin-HRP to minimize the nonspecific background. Plasma from one normal volunteer was also treated with an Albumin/IgG removal kit and included as control for cross-reactivity of antibodies with serum proteins. As a positive control, the same albumin/IgG depleted plasma sample was spiked with exogenous recombinant human sFasL.

6.12.3. PROTEIN ANALYSIS of sFASL BY HPLC

The supernatant from HEK cells transfected with the full-length extracellular domain of FasL cDNA expressing plasmid or supernatant from non-transfected cells were concentrated 46-fold. The supernatant proteins were separated according to the molecular weight using high-pressure liquid chromatography with a SynChropak GPC 100 column (250 mm x 4.6 mm; SynChrom) at a flow rate of 2 mL/min in PBS. The samples were separated into 80 fractions of 1 ml each. The column was calibrated by using alcohol dehydrogenase (150 kDa), bovine serum albumin (67 kDa), ovalbumin (43 kDa), chymotrypsinogen A (25 kDa), and ribonuclease A (13.7 kDa).

6.13. MICRO-LIQUID CHROMATOGRAPHY-ELECTROSPRAY IONIZATION TANDEM MASS SPECTROMETRY ANALYSIS

Protein samples containing human sFasL (Petrotech Inc.), previously exposed or not to the MPO-H_2O_2-Cl^- reaction, were incubated and denatured in 4 mM DTT, 50 mM ammonium bicarbonate pH 8.0, 10% acetonitrile for 15-20 minutes at 70-80ºC. After cooling to room temparature, proteins were alkylated by iodoacetamide (10mM) for 15 min; then digested with sequencing grade modified trypsin (Promega) (1:20 wt/wt, trypsin/protein) overnight at 37ºC. Digestion was halted by freezing the mixture or acidifying it (pH 2-3) with formic acid or trifluoroacetic acid. The micro-liquid chromatography-electrospray ionization tandem mass spectrometry (μLC-ESI-MS/MS) analysis was performed in the positive ion mode with a Finnigan LCQ Deca ProteomeX ion trap mass spectrometer (Thermo Electron Corp.) coupled to a quarternary Finnigan Surveyor HPLC (Thermo Electron Corp.). Tryptic peptides were separated using a reversed-phase capillary HPLC column (180 um x 10 cm; 5-μm particules; Biobasic 18, Thermo Electron Corp.) using solvent A (0,1% vol/vol formic acid in water) and solvent B (100% acetonitrile in 0.1% formic acid). The peptides were eluted using the following linear gradient: 0-10% B over 10 min; 10-35% B over 85 min; then 35-80% B over 5 min at a flow rate 2 μl/min. The MS/MS analysis was performed using 35% relative collision energy with an activation time of 30 min.

6.14. STATISTICAL ANALYSES

The results of the quantitative variables were expressed as media ± SEM, or plotted in scattered-dot graphs showing the median. To evaluate general differences between three or more groups, the analysis of variance (one-way ANOVA) followed by the Bonferroni's or Tukey post hoc tests was used for variables with normal distribution, or the Kruskal-Wallis followed by the Dunn's test for those without a normal distribution. A decimal logarithm (log_{10}) was used to reduce the heterogenity of variances when these were significantly different. A p value <0.05 was considered statistically significant. The values of the variables were saved in an Excel sheet, and the statistical analyses were performed using GraphPad Prism 5.0 (GraphPad Software Inc, San Diego, CA).

7. – RESULTS

Soluble Fas Ligand has been shown to increase in the BAL fluid of patients with ARDS [284, 468], and it is thought to play a role in the development of ALI from diverse causes. In the present studies, we have investigated the form of sFasL which is present in the lungs of patients with ARDS, the effects and mechanisms of injury of human sFasL in mouse lungs, and the structural factors of sFasL that determine its biological activity.

7.1. AGGREGATION STATUS OF HUMAN sFASL IN THE LUNG OF PATIENTS WITH ARDS

Previous studies have shown that sFasL is increased in the lung of patients with ARDS and is correlated with mortality [284, 468], but the aggregation status of sFasL in the lungs of patients with ARDS is not known. To explore this issue, we studied the BAL fluid obtained from four normal volunteers, four patients at-risk for ARDS and four patients with established ARDS using SDS-PAGE gel electrophoresis under non-reducing conditions followed by Western blotting. As shown in Figure 7.1, the BAL fluid from patients with ARDS contained a band at 50-kDa which corresponds to sFasL dimers (the molecular weight of sFasL after glycosylation is 25-kDa). Importantly, the 50-kDa band was not present in BAL fluid obtained from normal volunteers or from patients at risk (open arrow in Fig. 7.1). The absence of this band when control antibody was used confimed that the 50-kDa band in ARDS BAL fluid corresponded to sFasL (goat anti-V5 lanes in Fig. 7.1). It is possible that the 75-kDa and larger molecular weight bands represent higher aggregations of sFasL, but this could not be confirmed because some of these bands were also present with the control antibody, indicating intense cross reactivity with other high molecular weight proteins. In summary, the present results confirm that sFasL increases in BAL fluid from patients with ARDS, and that it tends to aggregate in the BAL fluid, suggesting that aggregation of sFasL has a pathogenic role in acute lung injury in humans.

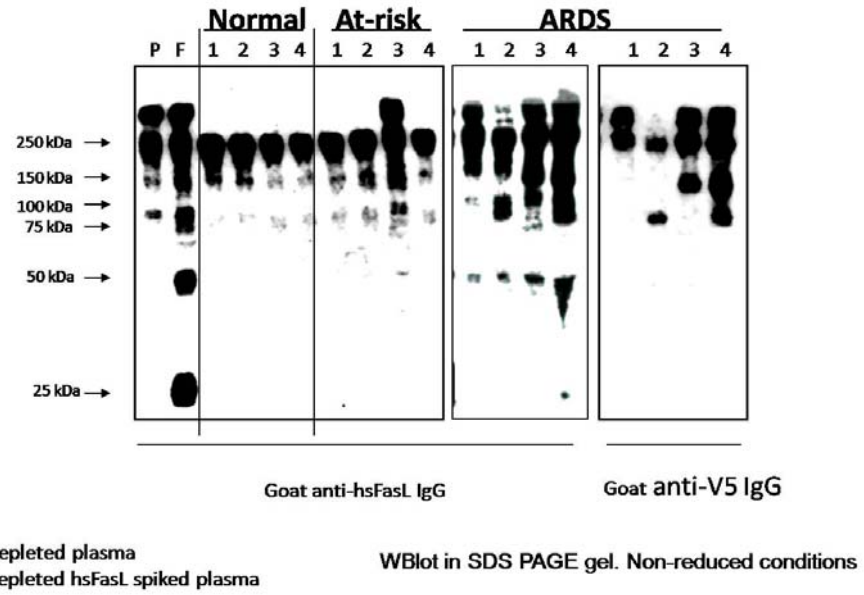


Figure 7.1. Soluble FasL (sFasL) increases in BAL fluid from patients with ARDS and tends to aggregate forming, at least, dimers.

IgG and albumin depleted BAL fluids from four normal volunteers, four patients at-risk for ARDS and four patients with established ARDS was loaded onto 4-12% SDS-PAGE gels under non-reducing conditions. Electrophoresis and Western blotting with a goat anti-hsFasL antibody or its isotype antibody control (goat anti-V5 IgG) were performed. IgG and albumin depleted plasma from one normal volunteer (Lane P) was included to control for cross-reactivity of antibodies with serum proteins. As a positive control, the same depleted plasma sample was spiked with exogenous recombinant human sFasL (Lane F). Each lane shows the BAL fluid of an individual patient and the arrows indicate molecular weight. Monomers of hsFasL have MW ~ 25 kDa.

7.2. BIOLOGICAL EFFECTS OF INTRATRACHEAL INSTILLATION OF sFASL IN MOUSE LUNGS

Previous studies from Matute-Bello and Martin (lab Mentors) showed that sFasL is active and induces apoptosis in Fas sensitive cells *in vitro* [284], but the relevance of sFasL for the pathogenesis of ALI *in vivo* is still controversial. To investigate in vivo the effects of sFasL, we performed a series of experiments testing the effects of different types of sFasL molecules administered via intratracheal instillation in mice.

7.2.1. Intratracheal instillation of human sFasL in mice induces lung injury via Fas activation

In order to study the effects of sFasL in an in vivo system, we first instilled recombinant mouse sFasL intratracheally to C57BL/6 mice, expecting that this would result in lung injury. Surprisingly, intratracheal instillation of mouse sFasL had no effects in mouse lungs (data not shown). Because of the poor biological activity of mouse sFasL, we then instilled recombinant human sFasL (rh-sFasL) into mouse lungs. In order to exclude the possibility that any responses to the human form of sFasL were due to the immune recognition of a human protein and not to the specific activation of Fas receptor by sFasL, we also tested a group of mice (*lpr* mice) in which the Fas receptor is not functional. C57BL/6 wild-type and *lpr* mice (n=10 per group) received a single intratracheal dose of human sFasL (25 ng/g) and the degree of lung injury was assessed at 16 h post-instillation. C57BL/6 wild-type mice that were either non-instilled (n=5) or intratracheally instilled with PBS (n=10) were used as negative controls for the experiments. To assess the degree of lung injury, we evaluated the presence of: i. structural alterations of the alveolar wall, ii. cell death, and iii. alveolar-capillary barrier dysfunction.

7.2.1.1 *Lung weight and structural alterations of the alveolar wall*

The lung wet weight was measured at the time of necropsy as an indicator of lung injury. Intratracheal instillation of human sFasL resulted in a significant increase of the lung wet weight in wild-type mice compared to normal mice and PBS-instillated controls. The increase of lung wet weight induced by instillation of human sFasL, however, was significantly attenuated in Fas-deficient *lpr* mice.

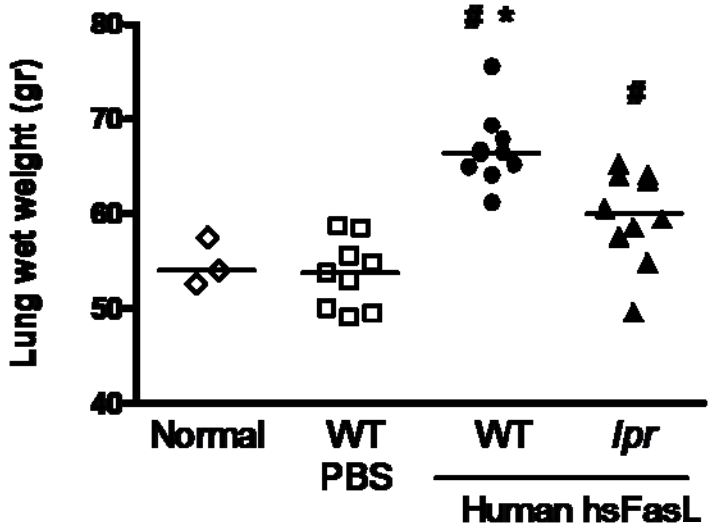


Figure 7.2. Lung wet weights of normal WT mice (Normal), and wild-type (WT) and *lpr* Fas-deficient (lpr) mice treated with PBS or recombinant human sFasL (25 ng/g) via intratracheal instillation. The left lung was removed and the lung wet weight was determined using an electronic balance (sensitivity, 0.0001 g). Each point represents an individual mouse. Horizontal bars represent the medians. Statistical analysis: one-way ANOVA with Bonferroni's post-hoc test. (#) $p<0.05$ vs PBS-WT, (*) $p<0.001$ vs human sFasL-lpr.

The effects of human sFasL instillation were also histologically evaluated in H&E stained sections of formalin-fixed lung tissues. In wild-type C57BL/6 mice (WT), human sFasL caused important lung tissue damage at 16 hours after intratracheal instillation that was characterized by the thickening of the alveolar wall, as well as by the presence of vascular congestion, alveolar hemorrhage and neutrophilic infiltratres. In contrast, the lung structure of the *lpr* mice harboring an inactive Fas receptor was normal (Fig. 7.3).

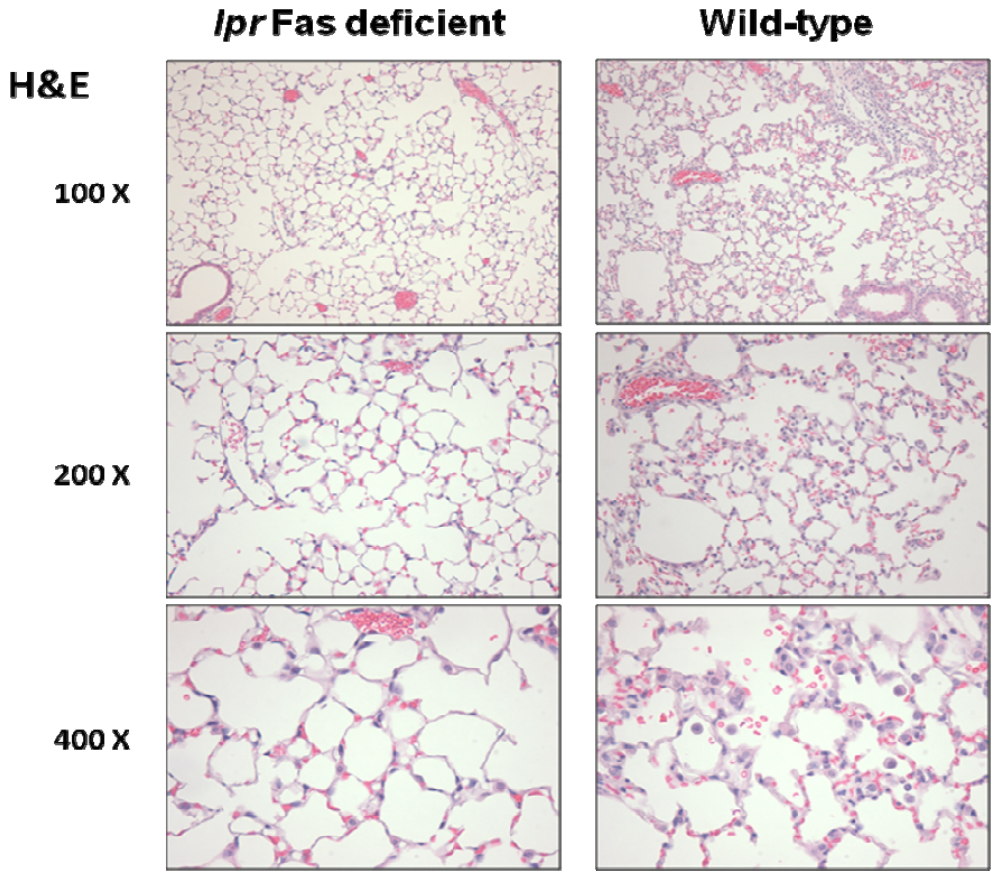


Figure 7.3. Representative lung tissue sections stained with hematoxylin-eosin from wild-type and Fas-deficient *lpr* obtained 16 hours after the intratracheal instillation of human sFasL (25 ng/g). The lungs from wild-type mice treated with human sFasL showed marked thickening of the alveolar walls, vascular congestion, neutrophil infiltration as well as deposition of proteinaceous material in the airspaces. The lungs from Fas-deficient *lpr* mice, in contrast, had normal appearance.

7.2.1.2. *Apoptotic cell death*

To determine whether the tissue changes were associated with apoptosis, we performed immunohistochemistry for the active (cleaved) form of caspase-3, a key intermediate in the Fas signaling pathway. In wild-type mice, the intratracheal instillation of human sFasL resulted in a significant increase in activated caspase-3 immunostaining localized to the alveolar walls as well as in some cells in the air alveolar spaces. In contrast to wild-type mice, caspase-3 activation was not detected in the lung tissue of Fas-deficient mice receiving human sFasL (Fig. 7.4)

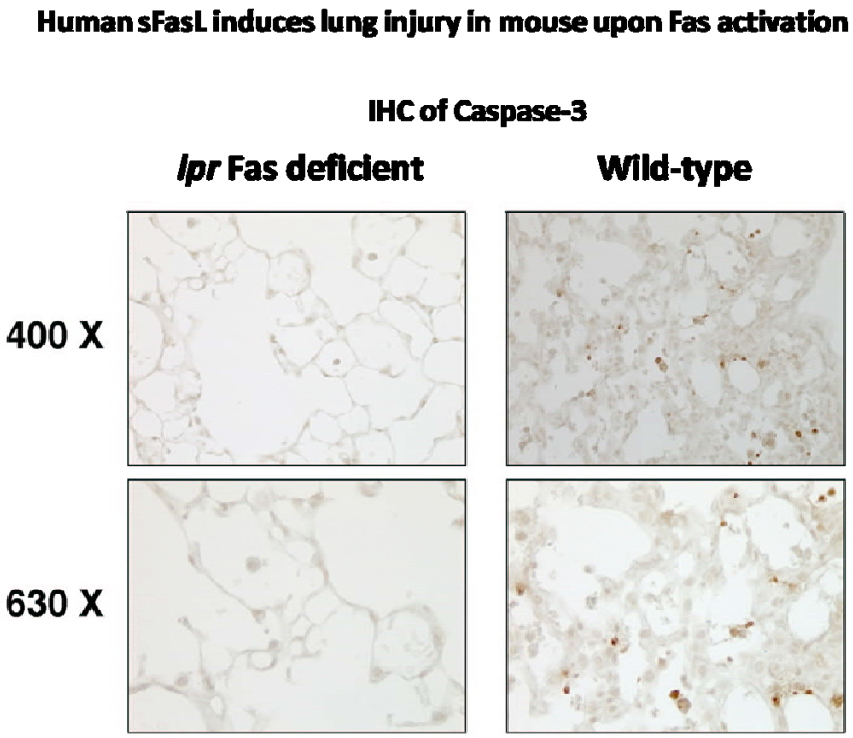


Figure 7.4. Immunohistochemistry of activated caspase-3 in lung tissue sections from wild-type and Fas-deficient *lpr* mice 16 hours after intratracheal instillation of human sFasL (25 ng/g). The lungs from wild-type mice treated with human sFasL showed increased immunostaining (brown reaction product) reflecting the presence of cleaved (active) caspase-3, which was mainly localized to the alveolar walls but also to some cells in the airspaces. In contrast, the lungs from Fas-deficient mice did not show any brown reaction product.

7.2.1.3. *Alveolar-capillary protein permeability*

At 16 hours after the intratracheal instillation of human sFasL, wild type mice presented a remarkable increase in the total protein concentration in BAL fluid (Fig. 7.5.A.). The BAL fluid concentration of IgM, which is a protein normally confined to the plasma compartment due to its large size (900 kDa), was also significantly elevated in wild-type (WT) mice receiving human sFasL compared to PBS instilled mice (Fig. 7.5.B.), suggesting an alteration of the permeability of the alveolar epithelial barrier. The BAL fluid concentrations of total protein and IgM were significantly lower in Fas deficient *lpr* mice compared to WT mice, although they were still increased compared to control mice receiving PBS.

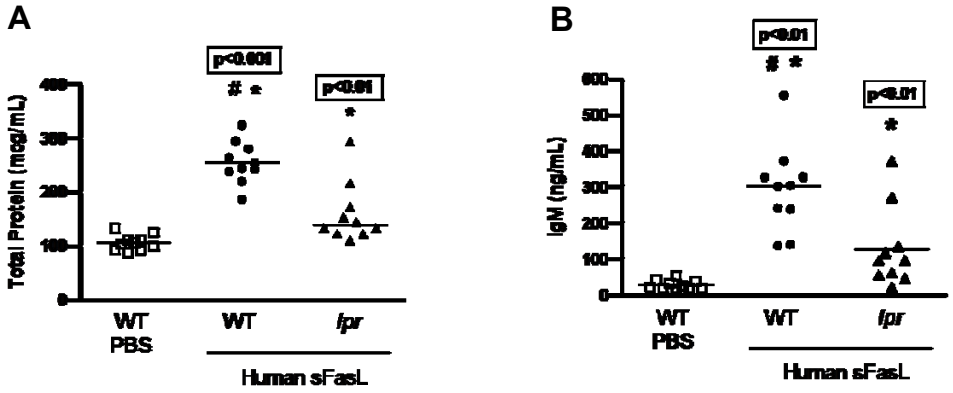


Figure 7.5. Concentrations of total proteins and IgM in BAL fluid of wild-type (WT) and Fas-deficient (*lpr*) mice treated with PBS or recombinant human sFasL (25 ng/g) via intratracheal instillation. BAL fluid was collected 16 hours later. Total proteins were measured by the BCA method (A), and the concentration of IgM was determined by ELISA (B). Results are shown as scattered dots where each point represents an individual mouse. Horizontal bars represent medians. Statistical analysis: one-way ANOVA with Bonferroni's post-hoc test. (#) vs human sFasL-*lpr* mice, (*) vs PBS-WT mice.

7.2.2. Mechanisms of sFasL-induced lung injury in mice

To study the potential mechanisms by which the intratracheal administration of recombinant human sFasL (rh-sFasL) caused lung injury in the mouse, we investigated the inflammatory responses (inflammatory cell recruitment and cytokine expression) and quantified the activation of pro-apoptotic pathways (caspase-3 activity) in the lungs at 16 hours after the instillation of rh-sFasL.

7.2.2.1. *Inflammatory cell recruitment*

Migration and activation of pro-inflammatory cells are important events in the pathogenesis of ALI from diverse causes. Neutrophils (polymorphonuclear neutrophils; PMN) predominate in the pulmonary edema fluid and bronchoalveolar lavage fluid obtained from patients with ALI. To investigate the inflammatory cell recruitment to the airspaces of the lungs induced by the instillation of human sFasL, we determined the number and type of white blood cells present in the BAL fluid collected from mouse lungs (Fig. 7.6). Compared

with mice receiving an intratracheal instillation of PBS, the BAL fluid from wild-type mice receiving human sFasL contained a significantly higher number of white blood cells, which were predominantly neutrophils (83%), but also macrophages (16%) and lymphocytes (1%). In BAL fluid from Fas-deficient *lpr* mice, however, rh-sFasL did not induce any elevation of the number of white blood cells, and all the cells present were mononuclear macrophages rather than PMN or lymphocytes. The instillation of human sFasL, therefore, resulted in significant recruitment of PMN and lymphocytes to the airspace only in wild-type mice with a functional Fas receptor.

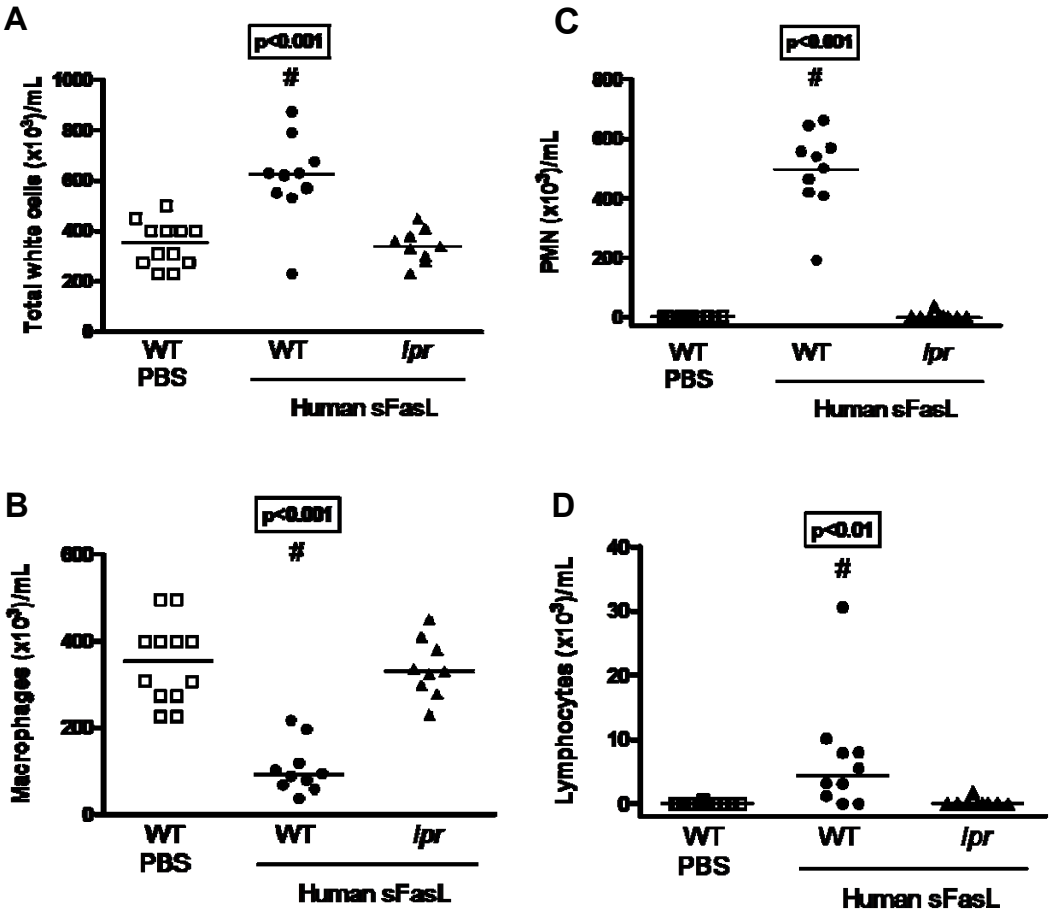


Figure 7.6. Total number of white blood cells (A), macrophages (B), PMN (C) and lymphocytes (D) in BAL fluid of wild-type (WT) and Fas-deficient (*lpr)* mice treated with PBS or human sFasL (25 ng/g bw) via intratracheal instillation. At 16 hours after the intratracheal administration of PBS or human sFasL, the BAL fluid was recovered from the mouse lungs, and it was immediately processed for total and differential cell counts using a

hemocytometer. The differential cell analysis was performed by Diff-Quick-stained cytospin preparation counting a total of 200 cells per slide in randomly selected high-powered fields (X1000). Administration of human sFasL into the mouse lungs caused a significant influx of inflammatory cells (predominantly PMN and, to a less extend, lymphocytes) into the airspace of wild-type mice expressing a functional Fas receptor. In contrast, no inflammatory cell recruitment occurred in lpr Fas-deficient mice. Each point represents one mouse. Horizontal bars are medians. Statistics: ANOVA with Bonferroni's post-hoc test. (#) vs human sFasL-*lpr* and PBS-WT mice.

To further confirm the rh-sFasL-induced recruitment of PMNs to the lungs, we measured the myeloperoxidase activity in whole lung homogenates. Myeloperoxidase (MPO) is a peroxidase enzyme that is abundantly present in neutrophils and to a lesser extent in monocytes. Thus, the MPO activity is frequently used as an indirect measurement of the total PMN cell content in tissues. The MPO enzyme has an important role in innate host defense, because it catalyzes the formation of hypochlorous acid (HOCl), a powerful oxidant with microbicidal properties. This may be pathogenetically relevant to our experimental model, because free extracellular MPO in tissues can also lead to inflammation and tissue destruction.

As shown in Figure 7.7, the intratracheal instillation of rh- sFasL resulted in an increase in the MPO activity in the lungs of wild-type (WT) mice as compared with PBS-instilled mice, but this response was not present in Fas-deficient (*lpr*) mice. The fact that human sFasL failed to increase the MPO activity in lungs and PMN in the BAL fluid of Fas-deficient *lpr* mice indicates that human sFasL requires the presence of a functional Fas receptor in the cell membranes in order to induce the migration of PMN from the microcirculation to the airspaces of the lungs.

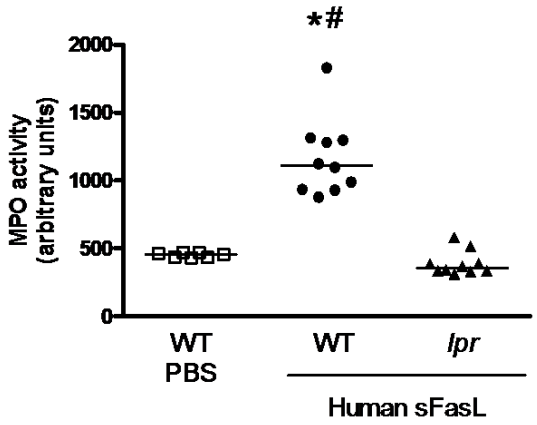


Figure 7.7. Myeloperoxidase activity in the lungs of wild-type (WT) and Fas-deficient (*lpr)* mice receiving an intratracheal instillation of PBS or human sFasL (25 ng/g bw). The myeloperoxidase (MPO) activity was measured using an enzymatic assay in total homogenates of lungs obtained from mice at 16 h after the intratracheal administration of PBS or human sFasL. Each point represents one mouse. The horizontal bars represent medians. Statistics: ANOVA with the Kruskal Wallis' post-hoc test. (#) $p < 0.05$ vs WT-PBS mice, (*) $p < 0.001$ vs human sFasL-*lpr* mice.

7.2.2.2. *Activation of inflammatory cytokines*

Cytokines include a group of proteins produced by a variety of cells. Cytokines can mediate normal cellular processes, and they are critical mediators of both innate and adaptive immune responses. Many cytokines are produced locally in the lungs by inflammatory cells, lung epithelial cells or fibroblasts at sites of injury, where these proteins initiate and amplify inflammatory responses. The concentrations of the pro-inflammatory cytokines IL-1β, KC and MIP-2 were measured in mouse lung homogenates at 16 hours after the intratracheal instillation of rh-sFasL. As shown in Figure 7.8, the concentrations of these three cytokines were significantly increased in the lungs of wild-type mice receiving rh-sFasL as compared with those receiving PBS. In contrast, rh-sFasL did not induce any increase of these cytokines in the lungs of Fas-deficient *lpr* mice. These results indicate that human sFasL induces the expression of pro-inflammatory cytokines in mouse lungs in vivo, and that such induction requires the presence of a functional Fas receptor.

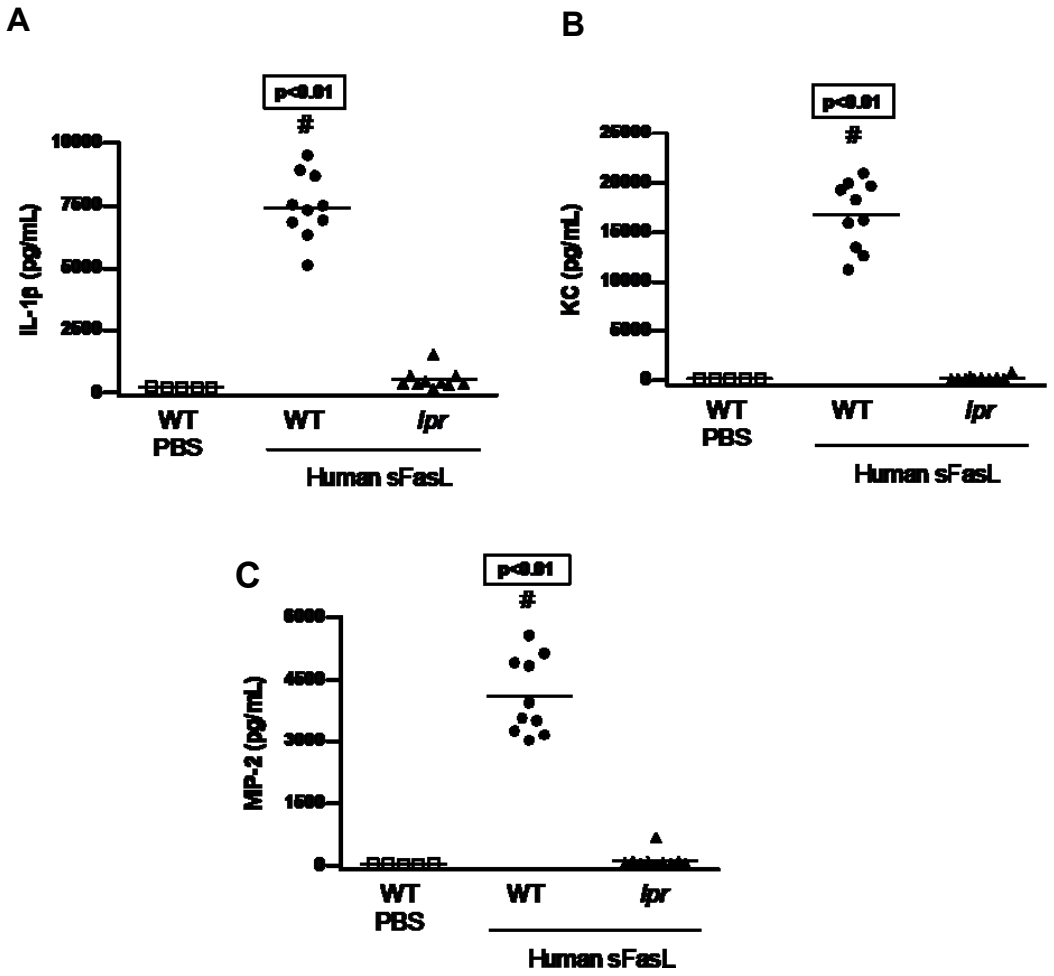


Figure 7.8. Human sFasL induced cytokine production in the lungs of wild-type mice (WT) but not in Fas-deficient mice (*lpr*). The concentrations of the proinflammatory cytokines IL-1β, KC and MIP-2 were measured in lung homogenates 16 hours after the intratracheal instillation of human sFasL using mouse-specific immunoassays (R&D Systems, Minneapolis, Minn.). The sensitivity of these immunoassays for IL-1β, KC and MIP-2 was 15.6 pg/ml. Each dot represents one mouse. The horizontal bars represent medians. Statistics: Kruskal-Wallis with Dunn's post-hoc test. (#) vs PBS-WT and human sFasL-*lpr* mice.

7.2.2.3. *Activation of pro-apoptotic pathways*

Previous studies showed that apoptotic pathways are activated in the lungs of patients with ARDS, and suggested that apoptosis is an important determinant of the fate of the lung epithelium in acute lung injury/ARDS. Activation of Fas mediates apoptosis via the activation of intracellular caspases which lead to the cleavage of nuclear DNA. Caspase-3 is an intracellular cysteine proteinase that exists in an inactive form as a proenzyme. Activation of procaspase-3 by

proteolytic processing is a key event required for apoptosis, and quantification of caspase-3 activity is commonly used as a measure of the extent of activation of apoptotic pathways.

Using an enzymatic assay, we quantified the activity of caspase-3 in lung tissues from wild-type and Fas-deficient *lpr* mice obtained at 16 hours after the intratracheal instillation of human sFasL. Wild-type mice receiving human sFasL had a significant increase in caspase-3 activity in the lungs, whereas there was no increase in caspase-3 activity in the lungs of Fas-deficient mice. These results indicate that human sFasL mediated the activation of caspase-3 specifically through the activation of the Fas receptor in mouse lungs (Fig. 7.9).

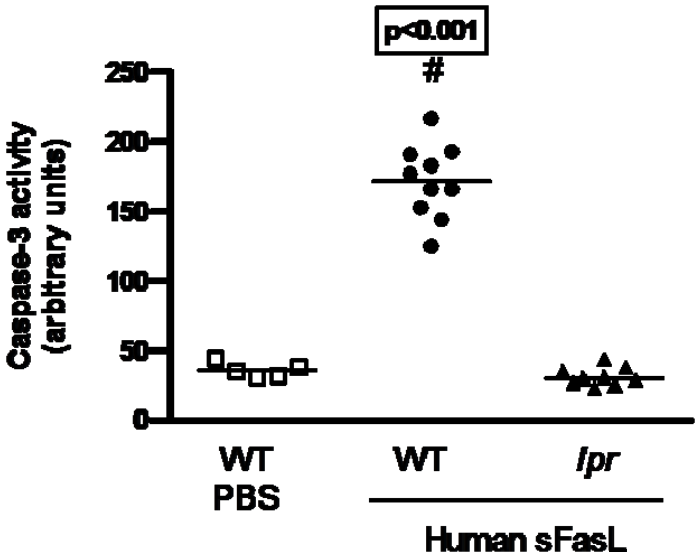


Figure 7.9. Human sFasL increases the activity of caspase-3 in the lungs of wild-type (WT) mice but not in Fas-deficient (*lpr*) mice. The WT and *lpr* Fas deficient mice were treated with intratracheal human sFasL from Alexis Co. (25 ng/g) and euthanized 16 hours later. Some WT mice treated with PBS were used as negative controls. In order to detect caspase-3 activity, the lysates of mouse lungs were incubated with (Z-DEVD)2-Rhodamince 110 substrate. Active caspase-3 cleaves a variety of molecules that contain the amino acid motif DEVD. The cleavage of this peptide releases Rhodamince 110 that when excited by light at 400 nm emits fluorescence at 505 nm. Caspase-3 activity is directly proportional to the fluorescence signal (505 nm) measured using a fluorescent microplate reader. Each dot represents one mouse. The horizontal bars represent medians. Statistical analysis: one-way ANOVA with Bonferroni's post-hoc test. (#) vs PBS-WT and human sFasL-*lpr* mice.

7.3. STRUCTURAL DETERMINANTS OF THE BIOLOGICAL ACTIVITY OF HUMAN sFASL

The sFasL detectable in BAL and edema fluids from patients with early ARDS is biologically active, suggesting that sFasL has a role at the onset of lung injury [284]. As shown previously (Fig. 7.1.), soluble FasL released into the air spaces tended to form aggregates in the BAL fluid of patients with ARDS. In the following experiments, our goal was to determine the structural determinants that control in vivo the biological activity of sFasL. The recombinant human sFasL (rh-sFasL) protein that induced the lung injury in mice in the previous studies comprises a TNF homology domain at the C-terminus, which mediates receptor binding and contains most of the trimerization domain, and a stalk region at the N-terminus, whose role is not yet understood. Therefore, we investigated the importance of aggregation and of the stalk region as potential determinants that may control the biological activity of human sFasL in the lungs.

7.3.1. N-terminal sequence, stalk region, and variants of sFasL

The two cleavage sites found at the extracelular sequence of the FasL imply that at least two forms of sFasL exist in vivo, a long form consisting of the stalk region and the TNF homology domain, and a short form consistent only of the TNF homology domain. To investigate the role of the stalk region at the N-terminus of the sFasL protein, we studied the biological activity and the degree of aggregation of different variants of recombinant human sFasL that were commercially available as well as some variants that were expressed in our laboratory (see Fig. 7.10.). In particular, we studied the following variants of human sFasL:

- Two recombinant long human sFasL proteins purchased from the Alexis and Peprotech companies. Both human sFasL proteins contain the same TNF homology [TNF(H)] sequence and stalk region but the Alexis form has a 26

amino acid leader sequence added by the company to the N-terminal stalk region. The Peprotech form, however, does not contain a the extra peptide.

- A short form of human FasL purchased from R&D Systems Co, which contains the TNF homology domain but lacks the stalk region, and has a 6-hystidine tail added at the N-terminus to facilitate purification.
- A long form of sFasL containing both the stalk and the TNF domain (aa 107-281 of human FasL; designated KH long human sFasL), and a short form of sFasL containing the TNF domain and only 6 aminoacids of the stalk region (aa 132-281 of human FasL; designated KH short human sFasL). These two forms of human sFasL were cloned and expressed in our laboratory, and they mimic the extracellular portions of FasL that can be processed by the action of metalloproteinases at the two identified cleavage sites. There is a 15 aa linker between Ig κ chain secretion signal cleavage site and cloned sFasL sequence. This 15 aa linker contains nonpolar amino acids (aside from 4 basic arginines) and lacks cysteine and methionines, so we predicted that it would not affect the biological activity or the comformational structure of the sFasL protein.

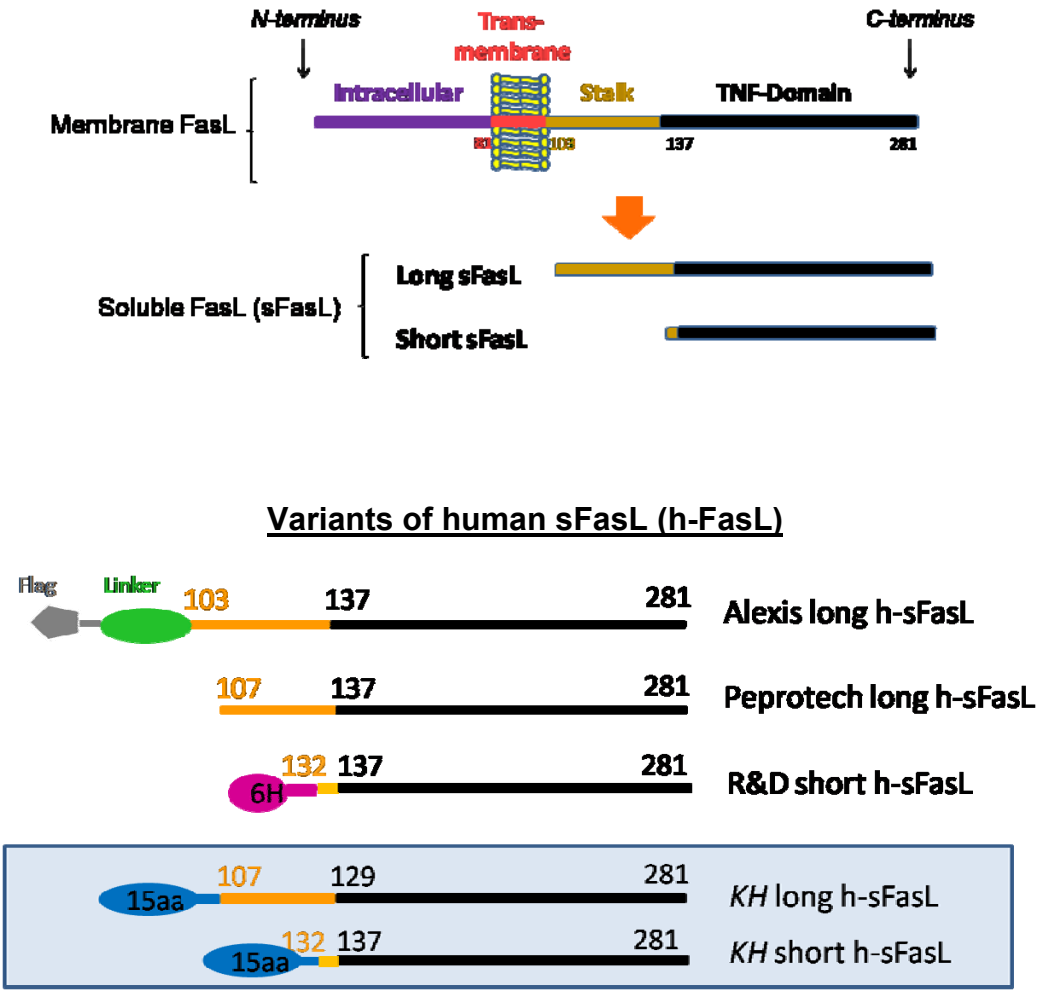


Figure 7.10. Variants of human sFasL with different N-terminal sequences.
The long forms of human sFasL from Peprotech and Alexis Co are all biologically active *in vitro* and *in vivo* in mouse lungs. Both proteins contain the stalk region, but the Alexis hsFasL also contains a cross-linking peptide and a FLAG tag at the N-terminus. The short form of sFasL from R&D System lacking the stalk region was inactive *in vitro* and *in vivo* in mouse lungs. This short form contains a 6-histidine residues at the N-terminus. A long and a short form of human sFasL were produced in our laboratory using the same expression and purification system (KH Long and KH short human sFasL). Both proteins had a leading 15 aa sequence contained in the expression vector to facilitate the expression and secretion of these proteins. TNF(H) = TNF-homology domain.

7.3.2. Level of aggregation of human sFasL variants

Prior studies have suggested that aggregation is required for the biological activity of sFasL in vitro [615, 619], but the importance of aggregation has not been tested in vivo. Interestingly, the sFasL released into the air spaces of patients with ARDS forms aggregates (see Fig. 7.1), suggesting that aggregation may be a factor that modulates the bioactivity of sFasL in vivo. To explore the structural factors determining the aggregation of human sFasL, we analyzed the degree of aggregation of the long and short forms of human sFasL (short and long KH human sFasL, cloned and affinity purified in our laboratory) using various techniques. The amino acid sequence (primary structure) is the main determinant of how a protein folds on itself and self-associates. To exclude the possibility that the extra 15-aa secretion peptide present at the N-terminus caused significant changes in the aggregation of our KH human sFasL proteins, we compared our forms of KH hsFasL to the commercially available long and short forms of human sFasL described in section 3.1, which have different amino acid sequences added at the N-terminus (see Fig. 7.10).

7.3.2.1. *Analysis by native and non-reducing SDS PAGE electrophoresis*

Electrophoresis in "native" or "non-denaturing" gels lacking sodium dodecyl sulfate (SDS) and reducing agents do not disrupt the covalent nor the non-covalent forces in the polypeptide backbone and between polypeptide units, preserving the natural protein folding and the degree of aggregation of proteins. Under these conditions, the long human sFasL proteins appeared as bands between 70- and 240-kDa, and the short forms appeared between 60- and 190-kDa (Fig. 7.11.A). Because the molecular weight of glycosylated monomers of long sFasL is 25-kDa and of short sFasL is 20-kDa, those bands corresponded to multimers of sFasL ranging from trimers (3 units) to nonamers (9 units). Bands at molecular weights higher than those corresponding to nonamers were also detected, particularly in the commercial proteins.

Many proteins aggregate by means of weak non-covalent forces between protein units, such as ionic or hydrophobic forces. To determine whether these non-covalent forces are involved in the aggregation of sFasL, we separated the proteins by electrophoresis in an SDS polyacrylamide gel under non-reducing conditions, and then analyzed the proteins by Western blotting (Fig. 7.11. B). The long sFasL proteins appeared as an intense band at 25-kDa and the short foms at 20-kDa, consistent with monomers. Less intense bands at 50- and 75-kDa were also observed, corresponding to dimers and trimers of sFasL, respectively.

Altogether, these data indicate that human sFasL spontaneously multimerizes in aqueous solutions and that, under these conditions, trimerization is its minimal level of multimerization. The data also suggest that the absence of the stalk region does not interfere with the propensity of sFasL to aggregate, as agreggation was also observed for the forms of sFasL lacking the stalk region (R&D short h-sFasL and KH short h-sFasL). After treatment with SDS, most of the human sFasL aggregates were changed into monomers and, in less extend, into dimers and trimers, indicating that high multimerization of human sFasL is due primarily to non-covalent forces. Covalent links between molecular units, however, may be involved in the formation of SDS-resistant dimers and trimers of sFasL.

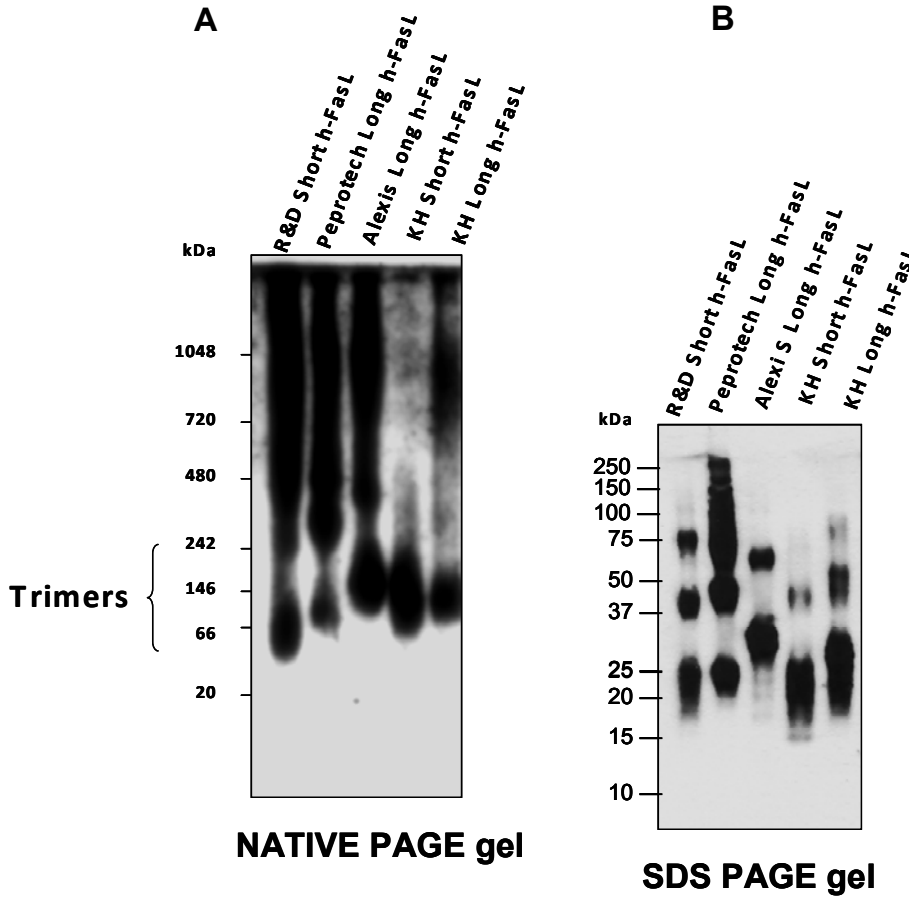


Figure 7.11. Aggregation of variants of human sFasL with different N-terminal sequences. Commercial and our homemade long and short forms of human sFasL were separated in non-reduced conditions by electrophoresis either in native (A) or SDS PAGE gels (B). Molecular weight of the proteins was determined by Western blot analysis using a polyclonal goat anti-human sFasL that recognizes both long and short sequences of human sFasL protein. In native gel, all variants of human sFasL regardless the presence or absence of the stalk region formed spontaneously trimers and larger multimers. In SDS gel, however, these variants of human sFasL were dissociated mainly to monomers.

7.3.2.2. *Analysis by HPLC*

Some proteins oxidize spontaneously during purification and storage, particularly those dissolved in aqueous solution without carrier proteins, and oxidation is a mechanism that induces protein aggregation. It was possible,

therefore, that the aggregation that we detected of our purified variants of human sFasL was induced by their oxidation and not by the intrinsic structural properties of the proteins. To minimize the potential effect of oxidation, we further analyzed the degree of aggregation of non-purified sFasL protein present in a protein-enriched solution. To achieve this, the non-purified long human sFasL present in the supernatant of HEK cells (in which sFasL cDNA coding the extracellular domain was transfected) was separated by size-exclusion chromatography, then each fraction was analyzed by the dot blotting (Fig 7.12.). The fractions containing more amount of sFasL protein, as confirmed by dot-blot analysis, corresponded to a molecular weight ranging form 52 kDa to larger than 158 kDa. This indicated that human sFasL forms aggregates ranging from dimers to aggregates larger than hexamers. By electrophoresis in SDS/PAGE gel, only one band was present at 25-kDa corresponding to monomers of human sFasL. These data indicate that the long human sFasL protein also forms aggregates in a protein-rich extracellular environment. These aggregates completely dissociated into monomers by SDS, suggesting that non-covalent forces were totally responsible for the aggregation of sFasL under these conditions.

HPLC of HEK cell supernatant containing human sFasL (KH long human sFasL)

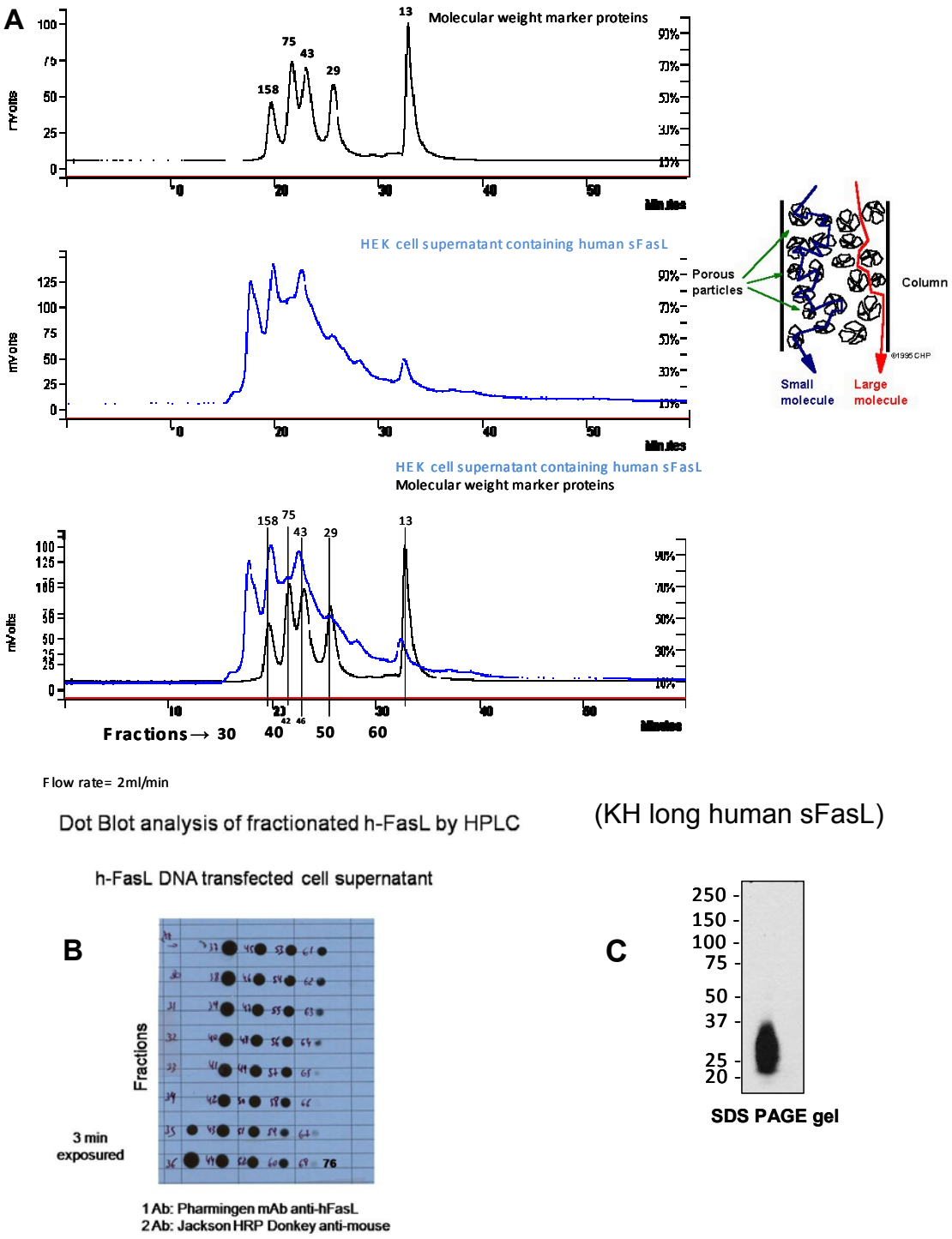


Figure 7.12. Analysis of HEK cell supernatants containing long sFasL using HPLC.

A) Cell supernatant from HEK cells transfected with cDNA encoding the extracellular portion of FasL was concentrated and proteins were separated by size exclusion HPLC at a flow rate of 2 ml/min in PBS (A). The supernatant sample was separated into 80 fractions of 1 mL each. The

column was calibrated using alcohol dehydrogenase (158 kDa), bovine serum albumin (67 kDa), ovalbumin (43 kDa), chymotrypsinogen A (25 kDa) and ribonuclease A (13.7 kDa) as showed in the black line. Separation of the KEH cell supernatant containing the sFasL is represented in the blue line. Molecular weights are also indicated. B) Dot-blot analysis of KH long human sFasL fractionated by HPLC. Although the KH long human sFasL was elutated from fraction 35 to 60, most of the protein was eluated between fractions 36 and 40 indicating that this long human sFasL aggregates mainly into trimers and larger molecular weights. C) The KH long human sFasL present in the HEK cell supernatant was totally dissociated to monomers when separated in electrophoresis in SDS PAGE gel in non-reducing conditions.

7.3.3. Role of the stalk region for the biological activity of human sFasL

The presence of two cleavage sites for the proteolytic action of matrix metalloproteinase 7 (MMP7, also known as matrilysin) at the extracellular sequence of FasL implies [488] that two forms of sFasL may exist in vivo. These forms of sFasL have two different predicted lengths and N-terminal sequences: i. a long sFasL containing the stalk region plus the TNF homology domain (if cleaved at the preferential cleavage site), and ii. a short sFasL comprising only the TNF domain (if cleaved at the alternative cleavage site).

We hypothesized that the long human sFaL, which contains the stalk region, was more active than its short form, and that MMP-7 could down-regulate the activity of long sFasL by releasing its stalk region. To test these hypotheses, we tested the biological activity of the long and short forms of human sFasL that we expressed in our lab (KH short and long human sFasL; Fig. 7.10) using the mouse in vivo model in which we previously demonstrated that human sFasL caused lung injury via Fas receptor activation. We also tested the cytotoxic activity of the two forms of human sFasL in human JKT cells, which are known to express Fas receptor in their cell membranes. Finally, the long human sFasL was treated with MMP-7 and the activity of the resultant product was tested in human JKT cells. With these experiments, we intended to give insight into the role of the stalk region on the biological activity of human sFasL.

7.3.3.1. *Stalk region and bioactivity of human sFasL: In vivo studies in mouse lungs*

In order to determine the role of the stalk region in the biological activity of sFasL in vivo, C57BL/6 mice (10 per group) were treated with a single intratracheal instillation (25 ng/g) of short or long human sFasL (KH short and long human sFasL). We assessed the degree of lung injury at 16 h post-instillation by measuring the lung wet weight, and by evaluating the presence of structural alterations of the alveolar wall and of alveolar-capillary barrier dysfunction. Organs from C57BL/6 wild-type mice (n=10) treated with intratracheal instillation of PBS were collected as controls for the in vivo experiments.

7.3.3.1.1. Lung injury induced by long or short human sFasL

7.3.3.1.1.1. Lung weight and structural alterations of the alveolar wall

As an indicator of lung injury, the total wet weight of the lungs was measured. Compared to PBS, instillation of long human sFasL resulted in a significant increase in the wet weight of the mouse lungs, but such increase was not observed in the mice treated with short human sFasL (Fig.7.13). This suggested that the long form of human sFasL had a higher potential than its short form to cause lung damage.

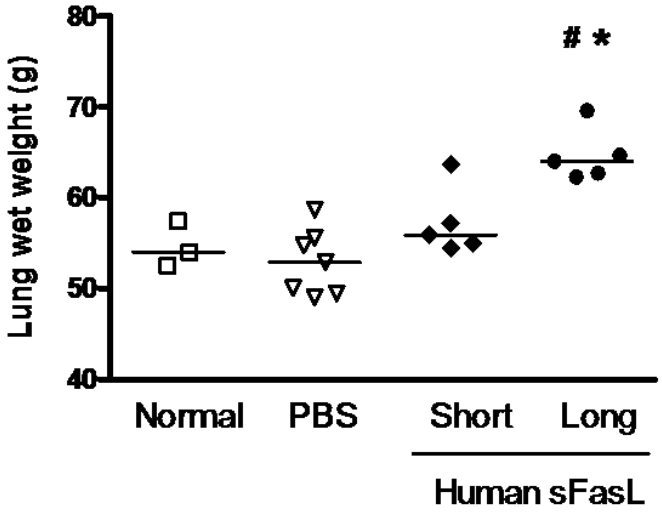


Figure 7.13. Lung wet weight of mice treated with a short and long form of human sFasL (25 ng/g) via intratracheal instillation. The left lungs were removed and the lung wet weight determined using an electronic balance (sensitivity, 0.0001 g). The lung wet weight of mice treated with the long form of human sFasL was significant higher as compared to PBS treated mice, whereas this effect did not occur in mice treated with the short form of human sFasL. The lung wet weight of normal mice (non-treated mice) was also included as negative control. Each dot represents individual mice. Horizontal bars represent medians. Statistical analysis: one-way ANOVA with Bonferroni's post-hoc test. (#) $p<0.001$ vs PBS, (*) $p<0.05$ vs short human FasL mice.

Lung tissue sections stained with H&E were used to evaluate the effects of the short and long forms of sFasL on mouse lungs with more detail. The lungs of mice receiving the long form of human sFasL showed alveolar wall thickening, vascular congestion, alveolar hemorrhage and neutrophilic infiltratres. In contrast, the lungs of mice treated with the short form of sFasL appeared normal (Fig. 7.14)

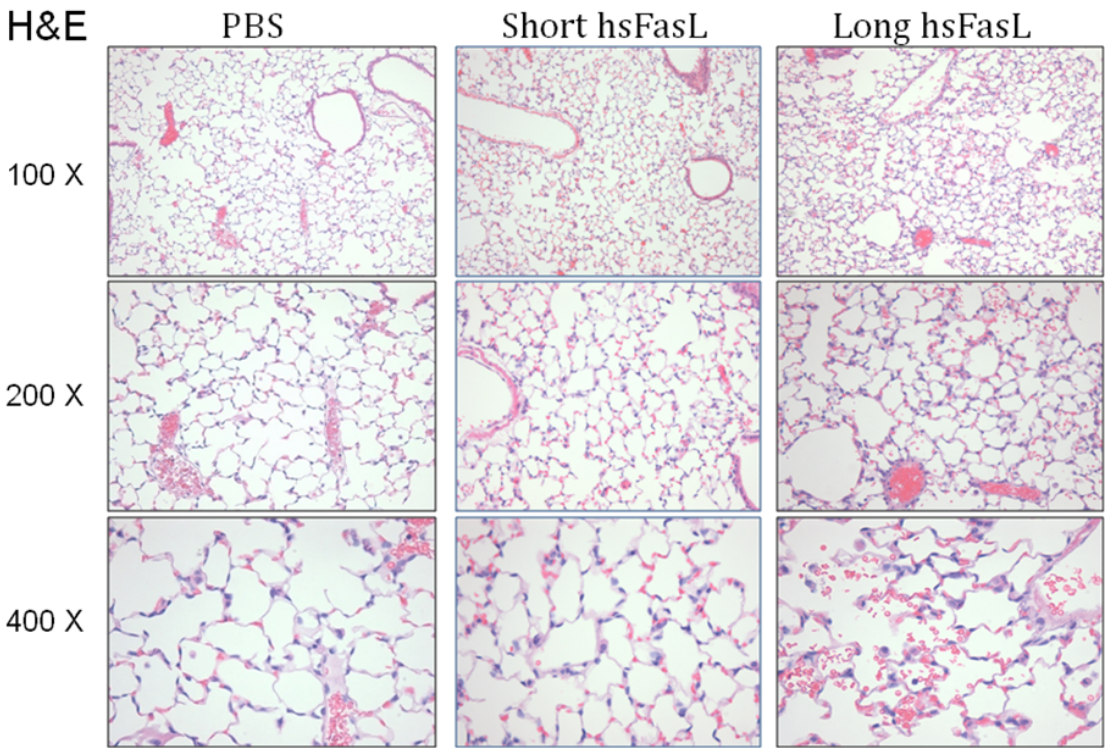


Figure 7.14. Representative lung tissue sections from mice at 16 hours after intratracheal instillation of the long or short form of human sFasL (25 ng/g). The lung from long hsFasL-treated mice showed marked thickening of the alveolar walls, vascular congestion and alveolar haemorrhage. In contrast, the lungs from short hsFasL-treated mice showed normal lung architecture, not different form mice treated only with PBS. This tissue sections were stained with hematoxylin-eosin.

7.3.3.1.1.2. Alveolar-capillary protein permeability

As shown in Figure 7.15, the concentrations of total protein and IgM were significantly increased in the BAL fluid of mice treated with long human sFasL. IgM is normally confined to plasma due to its large size (900 kDa), and its presence in BAL fluid suggests an increase in alveolar epithelial permeability. In the BAL fluid of mice receiving the short form of human sFasL, in contrast, the concentrations of total protein and IgM were similar to those observed in

mice receiving PBS. These results suggest that only the long form of human sFasL increased the permeability of the alveolar epithelial barrier.

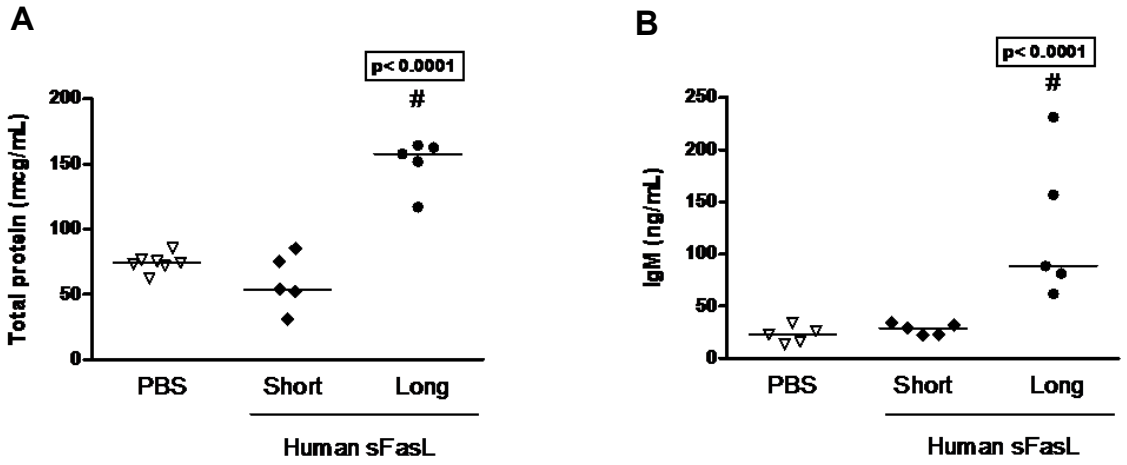


Figure 7.15. Concentrations of total protein and IgM in BAL fluid of C57BL/6 mice 16 hours after intratracheal instillation of the long or short forms of human sFasL (25 ng/g bw). The total proteins were measured using the BCA assay (A), and the concentration of IgM was determined by ELISA (B). Only mice treated with the long form of human sFasL had a significant increased in total proteins and IgM in the BAL fluid reflecting increase in protein permeability in the lungs. Each dot represents one mouse. The horizontal bars represent medians. Statistical analysis: one-way ANOVA with Tukey's post hoc test. (#) vs PBS and short human sFasL mice.

7.3.3.1.2. Mechanisms of lung injury induced by variants of human sFasL

7.3.3.1.2.1. Inflammatory cell recruitment

As shown in Figure 7.16, the BAL fluid of mice treated with long human sFasL presented a significant increase in the total number of white blood cells and PMN (60%), whereas the short form of human sFasL did not change the BAL cell profile compared to PBS instillation. In mice receiving long human sFasL, the number of PMN in the BAL fluid was significantly higher than in mice receiving either PBS or the short form of human sFasL, whereas the number of macrophages tended to decrease. Confirming these findings, the myeloperoxidase activity measured in total lung homogenates was significantly

increased only in the lungs of mice receiving the long form of human sFasL, but not in mice receiving the short form of human sFasL or PBS. These data indicate that only the long form of human sFasL is biologically active in vivo, promoting PMN recruitment and increasing lung epithelial permeability.

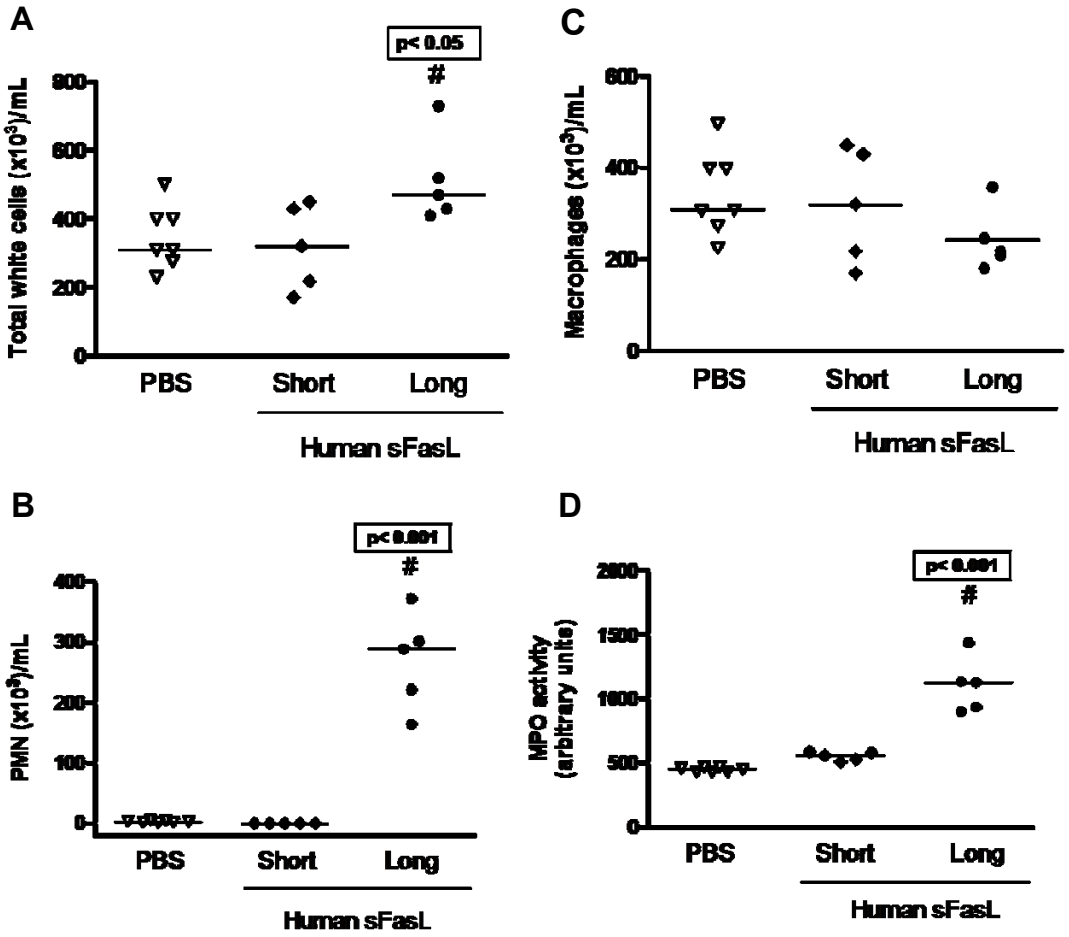


Figure 7.16. Cell counts in BAL fluid from C56BL/6 mice (A, B, C) and myeloperoxidase activity in total lung homogenates (D) at 16 hours after intratracheal instillation of either long or short forms of human sFasL (25 ng/g bw). Only mice treated with the long form of human sFasL had a significant increase in total white cells (A) and PMN (B) in the BAL fluid, and MPO activity in the lung homogenates. Each dot represents one mouse. The horizontal bars represent medians. Statistics: ANOVA with Tukeys' post-hoc test (#) vs PBS and short human sFasL mice.

7.3.3.1.2.2. Activation of inflammatory cytokines

Lung cytokine expression was assessed by measuring the concentrations of pro-inflammatory cytokines such as IL-1β, IL-6, KC, MIP-2, MCP-1, TNF-α, IFN-γ and IL-12, and anti-inflammatory cytokines such as IL-4 and IL-10 in lung homogenates (Fig. 7.17). At 16 hours after intratracheal instillation, the lungs of mice receiving long human sFasL contained high concentrations of IL-1β, IL-6, KC, MIP-2, MCP-1 IL-1β, MIP-2 and KC compared to mice receiving PBS, whereas the concentration of IL-4 was significantly decreased. The cytokines TNF-α, IFN-γ, IL-12 and IL-10 were undetectable in all groups. In contrast, mice receiving the short form of human sFasL had similar cytokine concentrations to mice receiving PBS, although they did have an increase in the anti-inflammatory cytokine IL-4.

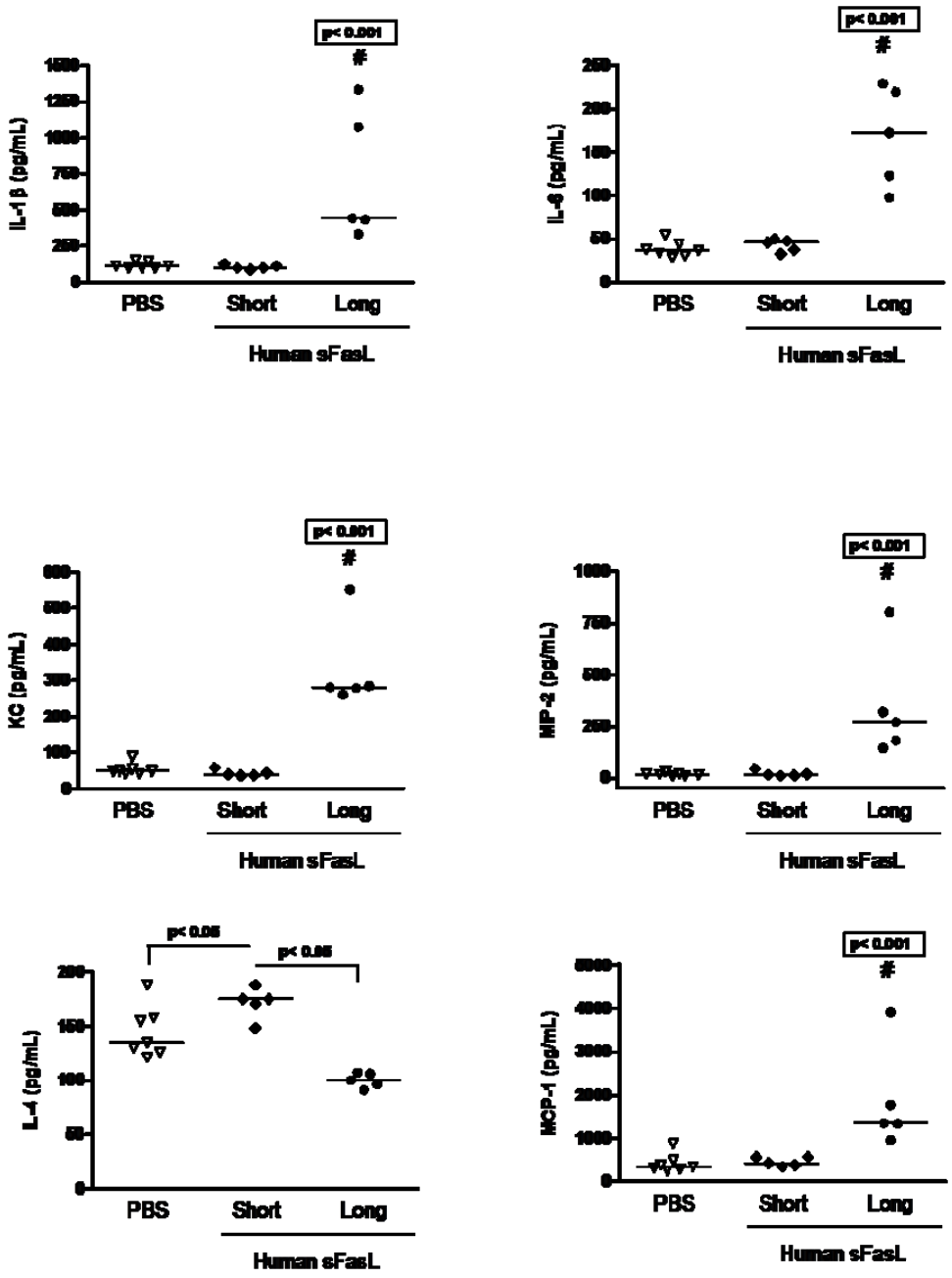


Figure 7.17. Cytokine production in total lungs of C57BL/6 mice treated with long or short human sFasL 16 hours after intratracheal instillation. The cytokine concentrations were measured in the lung homogenates by mouse-specific immunoassays (R&D Systems, Minneapolis, Minn.) using the LUMINEX system. Only mice treated with the long form of human sFasL had a significant increase in the expression of pro-inflammatory cytokines (IL-1β, IL-6, KC, MIP-2 and MCP-1) and a decrease in the anti-inflammatory IL-4. The sensitivities of these immunoassays were 37,9 pg/mL for IL-1β, 10,53 pg/mL for IL-6, 13.08 pg/mL for KC, 6.69 pg/mL for MIP-2 , 54.15 pg/mL for MCP-1, 6.82 pg/mL for TNF-α, 22.19 pg/mL for INF-δ, 11.25 pg/mL for IL-4, 7.47 pg/mL for IL-10. Each dot represents one mouse. The horizontal bars are medians. Statistics: ANOVA with Tukey's post-hoc test. (#) vs PBS and short human sFasL mice.

7.3.3.1.2.3. Activation of pro-apoptotic pathways

To evaluate the activation of pro-apoptotic pathways, we measured the activity of caspase-3 in lung homogenates. Instillation of long human sFasL induced a significant increase in caspase-3 activity in mouse lungs at 16 hours post-treatment as compared with mice treated with the short form of human sFasL or PBS alone. In mice receiving short human sFasL, the caspase-3 activity in the lungs was not different from that observed in mice receiving PBS (Fig. 7.18).

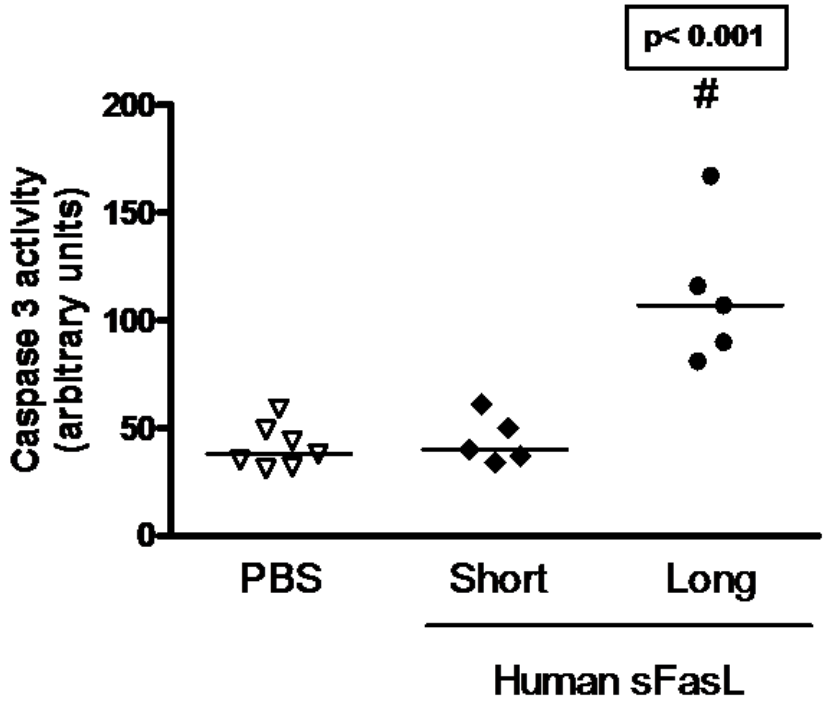


Figure 7.18. Pulmonary caspase-3 activity in C57BL/6 mice 16 hours after instillation of long or short human sFasL (25 ng/g). The lysates of mouse lungs were incubated with the (Z-DEVD)2-Rhodamince 110 substrate. Active caspase-3 cleaves proteins which contain the amino acid motif DEVD. The cleavage of this peptide releases Rhodamince 110, which when excited at 400 nm emits fluorescence at 505 nm. Caspase-3 activity is directly proportional to the fluorescence signal measured in a fluorescent microplate reader. Only mice treated with the long form of human sFasL had a significant increase in caspase-3 activity. Each dot represents one mouse. The horizontal bars represent medians. Statistical analysis: one-way ANOVA with Tukey's post-hoc test. (#) vs PBS and short human sFasL mice.

In summary, the increased bioactivity of the long form of human sFasL compared to its short form strongly suggest that the presence of the stalk region in the sFasL molecule is an important structural determinant of the effects of sFasL in the lungs in vivo.

7.3.3.2. *Stalk region and bioactivity of sFasL: In vitro studies in a human cell line*

To determine further whether the presence of the stalk region is related to the biological activity of sFasL, we tested the cytotoxic activity of the long and short forms of human sFasL in human JKT cells in vitro. The concentrations of both proteins were determined by ELISA using a polyclonal anti-human sFasL antibody that recognizes the two forms of the sFasL protein, as determined by western-bloting. Serial concentrations of both forms ranging from 12.3 ng to 1000 ng/mL were added to JKT cells. Exposure to long human sFasL induced significant cell death in JKT cells at a concentration of 12.3 ng/mL, as compared with cells treated only with media. The short form of human sFasL, however, did not show cytotoxicity until its concentration reached 111.1 ng/mL, which represents approximately a 10-fold lower cytotoxicity than the long form of human sFasL. Therefore, the presence of the stalk region in the N-terminus appears to be an important determinant required for the biological activity of human sFasL both in vivo and in vitro.

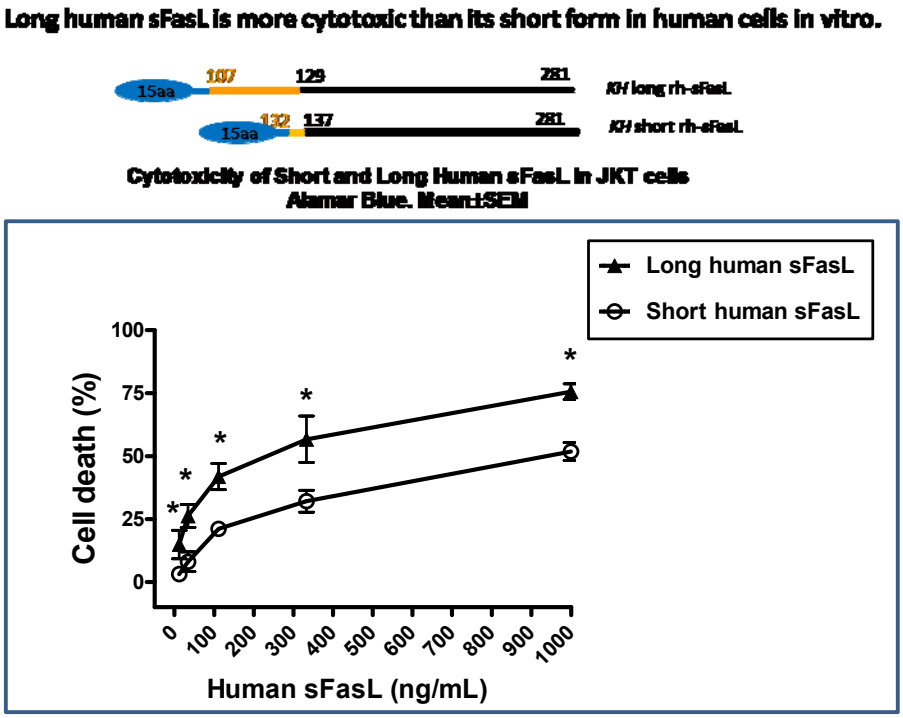


Figure 7.19. Cytotoxicity of long and short foms of human sFasL in JKT cells. Exposure of Jurkat cells to the long human sFasL led to a significant higher percentage of cell death compared to the exposure to the short form of human sFasL at all the concentrations tested. Results represent mean ±SD of 3 separate experiments performed in duplicates. One-way ANOVA with Tukey's post-hoc test. (*) $p<0.05$ vs short human sFasL.

7.3.3.3. *Proteolytic cleavage of the stalk region by MMP-7 and bioactivity of human sFasL*

The previous data strongly suggest that the stalk region at the N-terminal sequence is an important determinant of the activity of sFasL, as the short form of sFasL lacking the stalk region did not induce lung injury in mice, and it was less cytotoxic in human JKT cells in vitro compared with its long form. The amino acid sequence of long sFasL contains the "SL" cleavage sites near the C-terminus of the stalk region that are susceptible to cleavage by MMP-7. We hypothesized that cleavage of the long sFasL protein at the "SL" site by MMP-7 could release the stalk region and, thus transform the active long sFasL protein into the less active short sFasL form. To test this hypothesis, we incubated our

affinity purified long human sFasL protein together with human MMP-7 in an aqueous buffer. The size of sFasL cleaved by MMP-7 was determined by western-blot analysis and the cytotoxicity of the MMP-7 cleavage products was tested in JKT cells.

In the western-blot analysis with SDS PAGE gel, the untreated long human sFasL showed a prominent band at 25-kDa, corresponding to monomeric sFasL and less intense bands at 50-kDa and higher molecular weights corresponding to multimers. After treatment with MMP-7, the band corresponding to monomers shifted to a lower molecular weight, indicating that the polypeptide sequence of sFasL was shortened. The addition of MMP-7 was also associated with a decrease in the intensities of the 50-kDa and larger molecular weight bands, and with the appearance of a new band below 10-kDa that corresponded to the predicted molecular weight of the stalk region along with the extra 15-aa secreted peptide. The shift of the bands corresponding to monomers and the intensity of the band below 10-kDa increased as the dose of MMP-7 was increased. Cleavage at the SL site by MMP-7 with the consequent release of the stalk region likely account for the production of the band below 10-kDa and for the shift of the 25-kDa monomers to a lower molecular weight. Importantly, treatment with MMP-7 was associated with a reduction of the cytotoxicity of human sFasL in JKT cells. These results indicate that MMP-7 can process the stalk region of the sFasL in an aqueous solution, and that the release of the stalk region is associated with a reduction of the cytotoxic activity of sFasL. These results reveal for the first time the importance of the stalk region in determining the activity of human sFasL. Moreover, they also point to the proteolytic cleavage of sFasL by MMP-7 as a potential mechanism by which the cytotoxic activity of sFasL may be downregulated.

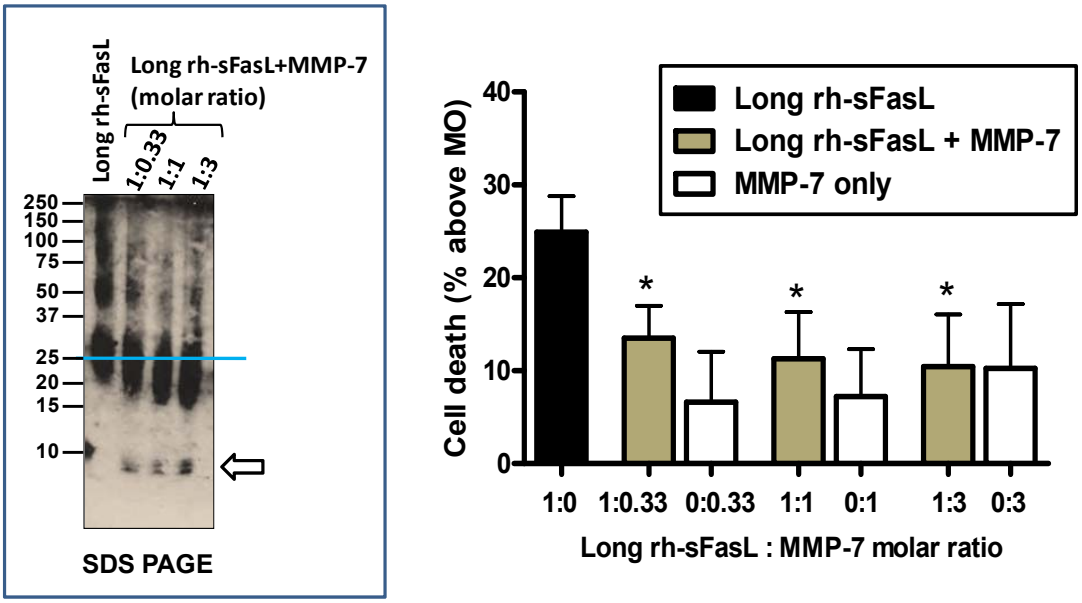


Figure 7.20. MMP-7 cleaves the long form of human sFasL and reduces its cytotoxicity in vitro. The long form of the recombinant human sFasL was incubated with serial concentrations of MMP-7 at the final human sFasL and MMP-7 molar ratios of 1:0.033, 1:1 and 1:3. The samples were subjected to SDS Page gel in non-reducing conditions, and also tested for cytotoxic using JKT cells. A) Western-blot of the KH human sFasL non-treated with MMP-7 (lane 1) or treated with serial concentrations of MMP-7 (lane 2, 3 and 4) using a polyclonal antibody for detection of human sFasL. Treatment with MMP-7 resulted in a shift of the monomers (25 kDa) to a lower molecular weight and in the appearance of a new band below 10-kDa (arrow) corresponding to the predictive molecular weight of the stalk region plus the 15 aa leading peptide. The horizontal bars indicate molecular weight. B) Cytotoxicity in Jurkat cells of the long human sFasL non-treated with MMP-7 used as a positive control (black bar), and MMP-7 alone used as the negative control (white bars). Incubation of long human sFasL with MMP-7 (grey bars) resulted in a significant decrease of the cytotoxicity in Jurkat cells, which was not significant different to the cytotoxicity of MMP-7 alone (white bars). These results suggest that MMP-7 can release the stalk region and reduce the biological activity of the long human sFasL in vitro. Results represent mean ±SD of three separate experiments performed in duplicate. One-way ANOVA with Tukey's post-hoc test. (*) $p<0.05$ vs Long human sFasL non-treated with MMP-7.

7.3.4. Role of an oxidative environment on the structure and bioactivity of human sFasL

Myeloperoxidase, H_2O_2 and reactive oxygen species (ROS) increase in the lungs of patients with ALI/ARDS [338, 632]. In the BAL fluid of these patients, many oxidized proteins accumulate in BAL fluid [423]. Oxidation modifies protein-protein interactions and promotes aggregation of proteins, often leading to significant changes in the protein activity [633-635]. In this study, we have found that the endogenous human sFasL present in the ALI/ARDS BAL fluid exits as covalent multimers (Fig 7.1). We have now investigated whether oxidants present in the lung fluid of patients with ARDS may account for the formation of covalent cross-links between polypeptide units of sFasL multimers and if they may even promote further multimerization or aggregation of this protein. Also, we evaluated whether oxidation modifies the biological activity of sFasL in vitro.

7.3.4.1. *Effects of BAL fluid from patients with ARDS on the multimerization of human sFasL*

The endogenous human sFasL present in the BAL fluid from patients with ARDS does not dissociate into monomers when exposed to SDS under non-reducing conditions (Fig 7.1). This resistance to SDS implies that covalent bonds are present between sFasL molecules. To determine whether substances present in the BAL fluid of patients with ARDS induce changes in the intermolecular forces between sFasL molecules promoting their multimerization, we incubated recombinant human sFasL (rh-sFasL) in BAL fluid pooled from normal subjects, from patients at-risk for ARDS, and from patients with established ARDS. For comparison, rh-sFasL was also incubated in PBS. The rh-sFasL was subsequently boiled and subjected to electrophoresis in SDS PAGE gel without reducing agents followed by Western blotting.

As shown in Figure 7.21, rh-sFasL incubated in PBS presented bands at 26- and 52-KDa, and a less intense band at 78-kDa consistent with the predicted molecular weight of glycosylated monomers, dimers and trimers, respectively. When rh-sFasL was incubated in BAL fluid from patients with ARDS, additional bands appeared at larger molecular weights ranging from 104-kDa to 250-kDa. These high molecular weight bands were not present, however, when rh-sFasL was incubated in BAL fluid from normal or at-risk subjects. The ARDS BAL fluid alone (without the addition of exogenous rh-sFasL) showed a band at 250 kDa that was not present in normal or at-risk BAL fluid alone. This 250 kDa band in the ARDS BAL fluid could be a result of nonspecific cross reactivity, although we cannot discard that such band represented the endogenous sFasL in an aggregated form. These data indicates that the BAL fluid of patients with ARDS promotes the multimerization of sFasL by establishing covalent forces between molecular units, as indicated by the resistance of those multimers to SDS and heat. Because rh-sFasL spontaneously forms large aggregates in basal conditions (Fig. 7.11), we cannot be certain that the 104- to 250-kDa multimers of the rh-FasL in the ARDS BAL fluid corresponded to further aggregation of this protein. Nevertheless, these data strongly suggest that there are substances present only in the BAL fluid of patients with ARDS, and not in normal subjects or patients at-risk that change the intermolecular forces and facilitate the formation of covalently linked stable aggregates of sFasL.

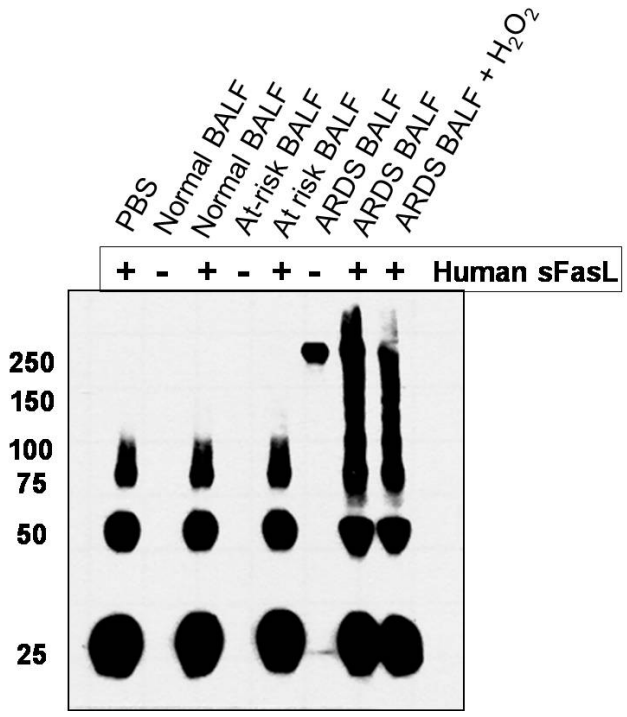


Figure 7.21. ARDS BAL fluid promotes covalent multimerization of exogenous human sFasL into larger molecular weight multimers compared to those present in normal or at-risk BAL fluid.

The BAL fluid from 3 normal volunteers, 3 patients at risk and 3 patients with ARDS were collected and pooled. Exogenous recombinant human sFasL (50 ng) was added to 10 ul of PBS or the pooled BAL fluid samples, and incubated for 90 minutes at 37ºC. The same volume of each BAL fluid samples without exogenous human sFasL protein was also incubated to be used as control. All samples (with and without exogenous sFasL) were boiled for 5 min and subjected to electrophoresis without reducing agents in SDS-PAGE gels. The molecular mass of the rh-sFasL protein was determined by Western blotting.

7.3.4.2. *Effects of the antioxidant L-methionine on the activity and multimerization of human sFasL in BAL fluid from patients with ARDS*

Next, we wanted to determine whether oxidation is a mechanism involved in the formation of covalent multimers of sFasL and whether it modifies the bioactivity of this protein in the BAL fluid of patients with ARDS. For this, we incubated exogenous recombinant human sFasL (rh-sFasL, from Peprotech Inc.) with different volumes (2, 4, 8 and 12 ul) of BAL fluid from patients with ARDS. At

the end of the incubation, we added free L-methionine in order to scavenge the potent oxidants such as HOCl. As a control, BAL fluid from patients with ARDS was incubated without rh-sFasL, which was incorporated immediately after the addition ofL-methionine to the reaction. The samples were then immediately subjected to SDS PAGE electrophoresis under non-reducing conditions, and the size of sFasL was analyzed by western blotting. The cytotoxicity of the samples containing 12 ul of BAL fluid with or without rh-sFasL was also tested in JKT cells.

Surprisingly, the rh-sFasL protein suffered important degradation when added to ARDS BAL fluids incubated previously with L-methionine (control samples), and such degradation was inversely proportional to the volume of ARDS BAL fluid. In contrast, the rh-sFasL incubated in ARDS BAL fluid without L-methionine (samples in which L-methionine was added at the end of the incubation), was not only prevented from suffering degradation but its covalent multimers were also better preserved as the volume of BAL fluid used was increased in the reaction. Compared to rh-sFasL in PBS, rh-sFasL incubated in ARDS BAL fluid induced slightly more cell death in JKT cells. This was not observed with the ARDS BAL fluid in which the rh-sFasL was added after L-methionine. These results suggest that oxidation by HOCl and other oxidants present in BAL fluid from patients with ARDS prevents the degradation and maintains the structural stability of rh-sFasL covalent multimers. Moreover, the incubation of sFasL with BAL fluid from these patients seemed to increase the cytotoxicity of sFasL in vitro. Together, these data indicate that sFasL is active in the lung fluid of patients with ARDS and that it is protected from degradation despite the presence of the elevated levels of oxidants and proteases that characterize such fluid. Moreover, oxidation seems to be a mechanism that prevents degradation and dissociation of sFasL multimers, whereas the addition of antioxidants makes the sFasL protein more susceptible to degradation in the ARDS BAL fluid.

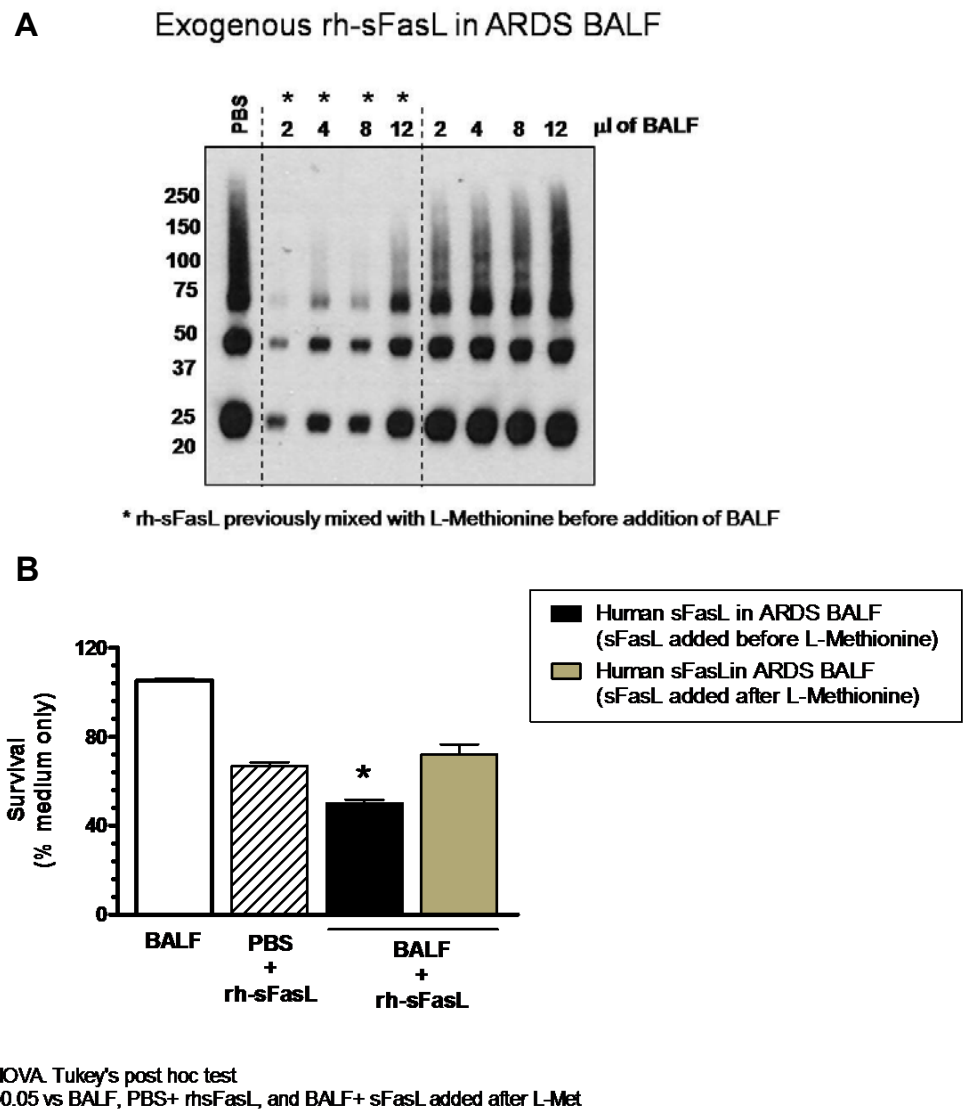


Figure 7.22. Western blotting (A) and bioactivity (B) of rh-sFasL incubated in BALF fluid of patients with ARDS. BAL fluid samples from 4 patients with ARDS were pooled and used undiluted. Exogenous rh-sFasL was incubated in PBS alone or with different volumes (2, 4, 8 and 12 ul) of ARDS BAL fluid pool at 37ºC for 90 min. At the end of the incubation, the HOCl scavanger L-methionine was added to the samples. As control, rh-sFasL was incorporated to the ARDS BAL fluid after the addition of L-methionine (*). A) After incubation, the samples were subjected to electrophoresis in SDS PAGE gel in non-reducing conditions and the size of the sFasL was analyzed by western blotting. B) The cytotoxicity of the samples containing 12 ul of BAL fluid with or without rh-sFasL was also tested in JKT cells in free serum media. Cell viability was detected by the Alamar Blue assay. JKT cells incubated with serum free media only and ARDS BAL fluid alone were used as negative control. Results represent mean ±SD of 3 separate experiments performed in duplicates.

7.3.4.3. *Effects of oxidation, nitration and chlorination on the multimerization of human sFasL*

Reactive oxigen species cause protein oxidation that can change protein conformation. This, in turn, can increase protein hydrophobicity and enhance protein-protein interactions leading to protein aggregation [634, 636]. Protein aggregation by oxidation can cause important changes in the biological activity of proteins. Hypochlorous acid (HOCl) is an oxidant that acts as a strong microbicidal agent, capable of killing bacteria, fungi and viruses. This oxidant also readily oxidizes proteins, causing changes in their activity and aggregation [637-640]. Neutrophils are a major source of HOCl, which is generated from hydrogen peroxide and chloride in a reaction catalysed by myeloperoxidase (MPO) [637, 641]. Both MPO and H_2O_2 are detectable in the lung fluid of patients with ARDS [338, 632].

To determine whether MPO-derived oxidants can induce covalent multimerization of human sFasL, we exposed recombinant human sFasL (rh-sFasL) to the MPO- H_2O_2-chloride or MPO- H_2O_2-nitrite systems using two different concentrations of H_2O_2. As shown in Figure 7.23, rh-sFasL formed covalent multimers only when it was incubated with MPO, H_2O_2 and NaCl together but not when incubated with either MPO- H_2O_2 or MPO-NaCl alone. On the other hand, the MPO- H_2O_2-nitration system had no effect on rh-sFasL covalent multimerization. These data suggest that reactive MPO products, but not reactive nitrogen species, promote the formation of covalent multimers of sFasL, and open the possibility that MPO-derived oxidants such as HOCl constitute a mechanism by which sFasL multimerizes in the BAL fluid of patients with ALI.

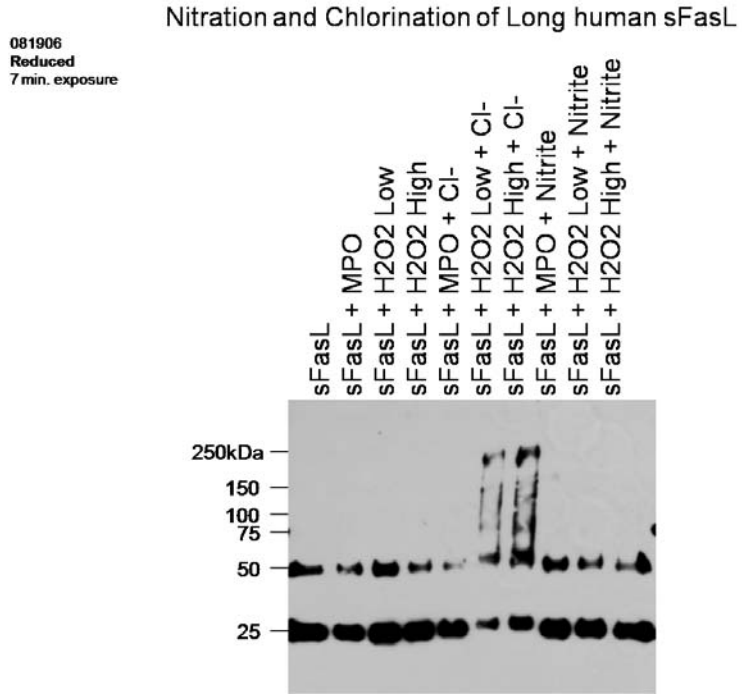


Figure 7.23. MPO-derived oxidation, but not nitration, enhances multimerization of rh-sFasL. rh-sFasL (50 ng) was incubated in PBS-DTPA alone or in the presence of two different concentrations of H_2O_2 (10 or 20 μM) supplemented with or without 50 nM MPO and either 100 μM NaNO2 or 100 mM NaCl. After 60 minutes, the reaction was terminated with 2.5 nM of L-Methionine. The molecular weight of rh-sFasL subespecies was determined by wentern-blotting analysis. The rh-sFasL covalently mulimerized when exposed to the three components of the MPO-H_2O_2-Chloride system. In contrast, the MPO-H_2O_2-Nitrate system had no effect on rh-sFasL multimerization.

7.3.4.4. *Effects of myeloperoxidase-derived HOCL on the multimerization of human sFasL*

To further confirm that MPO-derived HOCl changes the intermolecular forces and multimerizes sFasL, we incubated both human and mouse sFasL proteins with MPO, H_2O_2 and NaCl or directly with HOCl. The reaction was terminated by the addition of free L-Methionine, an HOCl scavenger. The proteins were incubated with the reducing agent 2-mercaptoethanol, heated at 96ºC, and separated by electrophoresis in SDS PAGE gel. Unlike recombinant human sFas, mouse sFasL forms only monomers in basal conditions (determined in

native gel, Fig. 7.24. A.1). As shown in Figure 7.24, mouse sFasL exposed to oxidation by the MPO system or HOCl multimerized into dimers, trimers and larger multimers. Human sFasL exposed to oxidation showed bands at the molecular weights corresponding to trimers and larger multimers. Both mouse and human sFasL multimers appeared in a dose-dependent manner with respect to the concentrations of H_2O_2, MPO, and HOCl. Pre-incubation with the HOCl scavenger L-Methionine completely prevented multimerization of human and mouse sFasL in the MPO reaction or with HOCl. When high concentrations of these two reagents were used, partial or total loss of protein detection occurred. In general, under these reducing conditions (2-ME, heat and SDS), the disulfide bonds and the ionic forces of the proteins are broken, so only protein aggregates formed by non-disulfide covalent bonds can persist. This indicates, therefore, that multimers of rh-sFasL observed under these reducing conditions can only be formed by non-disulfide covalent bonds. Taken together, these results indicate that oxidation by HOCL facilitates multimerization of sFasL by the formation of non-disulfide covalent bonds between molecular units.

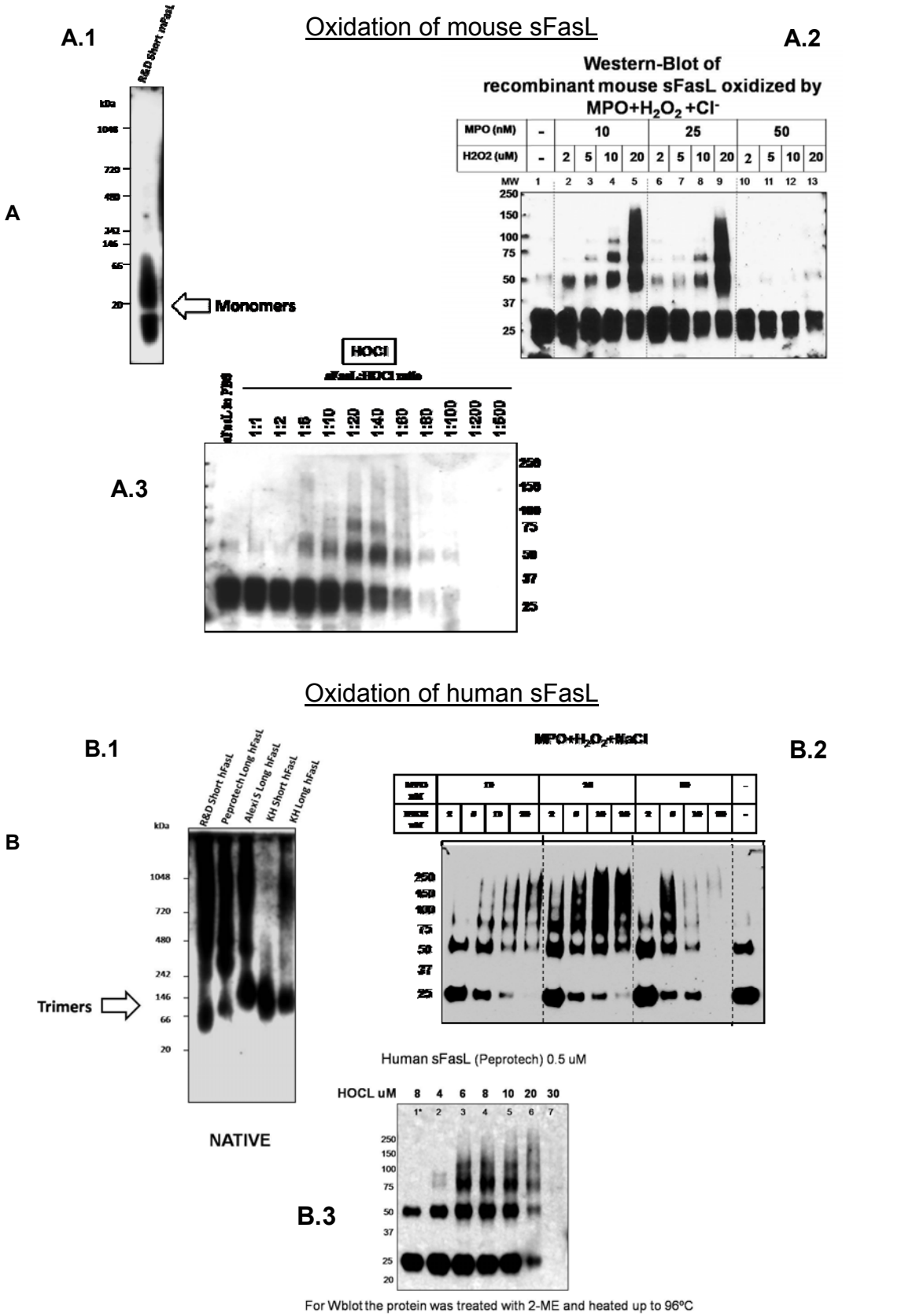


Figure 7.24. Incubation with MPO-derived oxidants or directly with HOCl promotes multimerization of sFasL. Recombinant mouse sFasL from the R&D systems Co and human

sFasL from the Peprotech Co diluted in PBS were subjected to electrophoresis in native gel followed by western blot analysis (arrow). The mouse sFasL forms only monomers (A.1.) whereas the human sFasL forms trimers and higher molecular aggregates in native non-denaturing gel in basal conditions (B.1.). We incubated these mouse and human sFasL proteins in PBS-DTPA alone or with different concentrations of MPO (10, 25, 50 nM) and H_2O_2 (2, 5, 20, 30 μM) plus 100 μM of NaCl (A.2 and B.2). Also, both proteins were incubated in PBS alone or with increasing concentrations of HOCl (A.3 and B.3). Exposure to MPO-derived oxidants induced multimerization of mouse and human sFasL in a dose-dependent fashion, although total or partial loss of protein detection was also observed with the highest concentrations of oxidants. Pre-incubation with the HOCl scavenger L-Methionine completely prevented multimerization and loss of detection of the sFasL protein incubated with HOCl (A.3)

7.3.4.5. *Role of the methionine residues on the response of sFasL to an oxidative environment*

Methionine and cysteine residues are oxidized very rapidly even under mild oxidative conditions due to the presence of sulfur in their side-chains. Oxidation of methionine residues is a mechanism by which the activity of proteins can be modified [634, 642, 643]. Curiously, human sFasL contains a considerably higher number of methionine residues than its mouse molecule counterpart (eight versus two, respectively), and this constitutes the main difference in the amino acid sequence between both proteins.

7.3.4.5.2. Influence of methionine residues on the multimerization of sFasL induced by oxidative conditions

To determine whether methionine is involved in the multimerization of sFasL under oxidative conditions, we took advantage of the fact that murine sFasL contains considerably fewer methionine residues than human sFasL and used molecular methods to produce a murine sFasL that contained methionines in the same positions as in human sFasL. For this, we first cloned mouse sFasL, and then we used site-directed mutagenesis to substitute only the amino acids

matching the location of methionine residues in human sFasL. We expressed a "wild-type" mouse sFasL with its two original methionine residues and a "humanized" met-mouse sFasL that contained a total of seven methionine residues. As shown in Figure 7.25, when these proteins were subjected to mild oxidation with HOCl, the met-mouse sFasL multimerized more readily than the native mouse sFasL. Multimerization did not occur, however, when oxidation of sFasL was prevented by the prior addition of free L-methionine to scavenge HOCl. All of these data strongly support that the methionine residues of sFasL are key determinants explaining the multimerization-promoting effects of an oxidative environment.

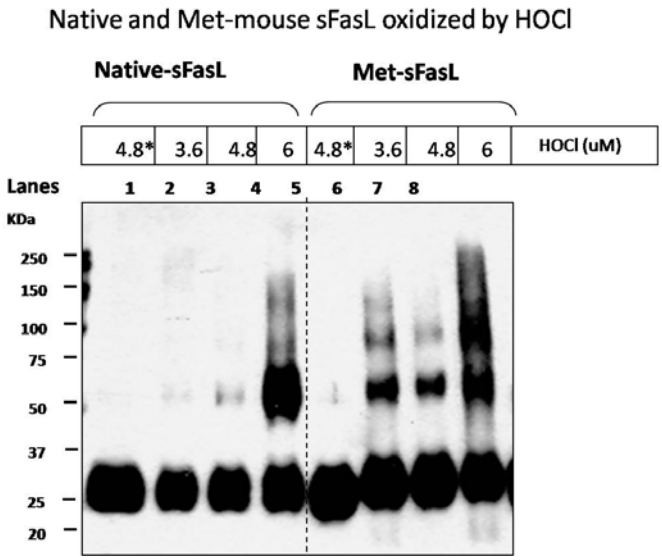


Figure 7.25. Addition of methionine residues in sFasL enhanced multmerization upon mild oxidation. By using site-directed mutagenesis, the number of methionine residues were increased in mouse FasL from two (native-sFasL) to seven (met-sFasL) as in human sFasL. Addition of methionine residues to mouse sFasL (lanes 6 to 8) enhanced multmerization with mild oxidation compared to its native form (lanes 2 to 4). As a control, scavenging HOCl with excess of free L-Methionine prevented multimerization of sFasL (lanes 1 and 5).

7.3.4.5.3. Extent of oxidation and identification of amino acids susceptible to oxidation in human sFasL protein

Most reactive oxygen species (ROS) can oxidize methionine (Met) residues of proteins to methionine sulfoxide (MetO) [634, 642, 643]. To investigate whether methionine oxidation is associated with the multimerization and activity of sFasL, we analyzed the rh-sFasL protein from the Peprotech Co. by mass spectrometry after exposing it to the MPO system. For this, the rh-sFasL protein was incubated in an aerobic atmosphere in an aqueous solution with or without the MPO+H_2O_2+NaCl reaction. The purified recombinant human sFasL was then digested with trypsin and the tryptic peptides were analyzed by liquid chromatography-electrospray ionization followed by tandem mass spectrometry (LC-ESI-MS/MS). With this method, the following ionized fractions were separated according to their mass (m)- to- charge (z) ratios (m/z) and the sequences were also determined:
ESTSQMHTASSLEK (m/z, 768.5),
SMPLEWEDTYGIVLLSGVK (m/z, 1069.3),
VYMR (m/z, 284.6),
YPQDLVMMEGK (m/z, 656.1),
MMSYCTTGQMWAR (m/z, 812.1).

Analysis by MS/MS of the human sFasL protein not exposed to the MPO-derived oxidant showed that the peptide fractions ESTSQMHTASSLEK and MMSYCTTGQMWAR appeared in major peaks with a mass to charge ratio of 776.4 and 820.1, respectively, suggesting that the molecular weight of the original peptides had increased by 8 Da, corresponding to the gain of one oxygen atom in each peptide. MS/MS analysis of these two modified peptides indicated that Met^{15} and Met^{132} of human sFasL are partly oxidized to methionine sulfoxide in an aqueous liquid even when no oxidants other than the oxygen molecules of H_2O were present. After oxidation by MPO-derived oxidants, MS/MS showed that ESTSQMHTASSLEK, SMPLEWEDTYGIVLLSGVK and VYMR peptides had increased by 8 Da (m/z,

776.4, 1077.1, 292.7 respectively), YPQDLVMMEGK by 16 Da (m/z, 672.1) and MMSYCTTGQMWAR by 24 Da (m/z, 836.0) corresponding to the gain of one, two or three oxygen atoms. MS/MS analysis of these modified peptides indicated that each methionine residue in the human sFasL sequence was partially oxidized to methionine sulfoxide. None of the other amino acid residues were oxidized, and chlorination of tyrosine was not detected.

These data indicate that spontaneous oxidation of two methionine residues (Met^{15} and Met^{132}) occurred when human sFasL was reconstituted in water in the absence of oxidant scavengers and handled in an aerobic atmosphere. Previous studies have shown that surface-exposed Met residues of proteins are major targets of oxidation. Therefore, it is possible that Met15 and Met132 might be located in the surface of the protein and be more susceptible to oxidation. Interestingly, all of the methionine residues of human sFasL, including Met^{15} and Met^{132}, were oxidized when exposed to MPO-derived HOCl. In contrast, other amino acids susceptible to oxidation, such as cysteine, tryptophan and tyrosine, remained unmodified, indicating that the methionine residues of sFasL are the main target for oxidation.

Analysis by mass spectrometry

Human sFasL in H20						
position	sequence	1	2	3	RT	Peak area (%)
10-23	ESTSQMHTASSLEK		768.5	512.6	29.5	98.5
	ESTSQM(O)HTASSLEK		776.4	517.9	26.8	1.5
51-69	SMPLEWEDTYGIVLLSGVK		1069.3		57.3	100
112-122	YPQDLVMMEGK	1310.5	656.1		41.4	100
123-135	MMSYCTTGQMWAR	1622.6	812.1		41.5	87.4
	MMSYCTTGQM(O)WAR	1638.6	820.1		40	12.6
105-108	VYMR	568.3	284.6		25	100

Met[15] and Met[132] of human sFasL are partly oxidized to methionine sulfoxide.

Human sFasL oxidized by MPO+H_2O_2*Cl-						
position	sequence	+1	+2	+3	RT	peptide (%)
10-23	ESTSQMHTASSLEK		768.5	512.6	42.53	59.4
	ESTSQM(O)HTASSLEK	1551.7	776.4	517.9	28.5	40.6
51-69	SMPLEWEDTYGIVLLSGVK		1069.3		57.53	45.9
	SM(O)PLEWEDTYGIVLLSGVK		1077.1		56.0	54.1
112-122	YPQDLVMMEGK	1310.5	656.1		42.2	46.3
	YPQDLVM(O)M(O)EGK	1342.6	672.1		36.0	53.7
123-135	MMSYCTTGQMWAR	1622.6	812.1		42.5	10.4
	M(O)M(O)SYCTTGQM(O)WAR	1670.6	836.0		36.04	13.6
105-108	VYMR	568.3	284.6		25.81	26.4
	VYM(O)R	584.3	292.7		23.10	73.6

All Met residues of human sFasL are partly oxidized to methionine sulfoxide by MPO+H_2O_2+Cl-

Figure 7.26. MPO-derived oxidants induce oxidation of methionine residues in sFasL.
MS and MS/MS analysis of peptides in a tryptic digest of recombinant human sFasL dissolved in H_2O (panel A) or exposed to the MPO system (panel B). The following peptides were analyzed: ESTSQMHTASSLEK (m/z, 768.5), SMPLEWEDTYGIVLLSGVK (m/z, 1069.3), VYMR (m/z, 284.6), YPQDLVMMEGK (m/z, 656.1), MMSYCTTGQMWAR (m/z, 812.1). The rh-sFasL dissolved in H_2O only had two methionines (Met[15] and Met[132]) partly oxidized to methionine sulfoxide. After exposure to the MPO system, however, the eight methionine residues present in the amino acid sequence of rh-sFasL were partly oxidized to methionine sulfoxide.

7.3.4.5.4. Relation between the extent of oxidation and the cytotoxic activity of human sFasL

Methionine oxidation can lead to changes in hydrophobicity, alterations in protein conformation and target proteins for proteolytic degradation [636]. The consequences of methionine oxidation in the biological activity of the proteins are variable and depend on the position of the methionine residues in the protein. Whereas oxidation of Met residues leads to loss of biological function in some proteins, in other proteins it can have little or no effect on their biological function. Recently, it has been demostrated that methionine oxidation

can increase pre-existing activities of proteins by facilitating the exposure of previously hidden binding sites [634, 642, 643].

To evaluate the effects of methionine oxidation on the biological activity of human sFasL, we exposed rh-sFasL (from the Peprotech Inc.) to MPO-H_2O_2-Chloride system, and compared the activity of the spontaneously oxidized rh-sFasL (with Met^{15} and Met^{132} oxidized) with the activity of the rh-sFasL exposed to the MPO oxidative reaction. The biological activity was tested in JKT cells in vitro, and cell survival was determined by Alamar Blue assay. Compared with the sponstaneusly oxidized rh-sFasL, the cytotoxic activity of rh-sFasL exposed to the MPO-H_2O_2-Chloride system diminished in a H_2O_2 dependent manner leading to an increased survival of JKT cells (Fig. 7.27). Preincubation with oxidant scavengers prevented the loss of the biological activity of rh-sFasL mediated by the MPO-derived oxidants. These data suggested that the spontaneous oxidation of Met^{15} and Met^{132} has little or no effect on the biological activity of human sFasL protein. In contrast, additional methionine oxidation by MPO-derived HOCl resulted in an important reduction of the biological activity of sFasL.

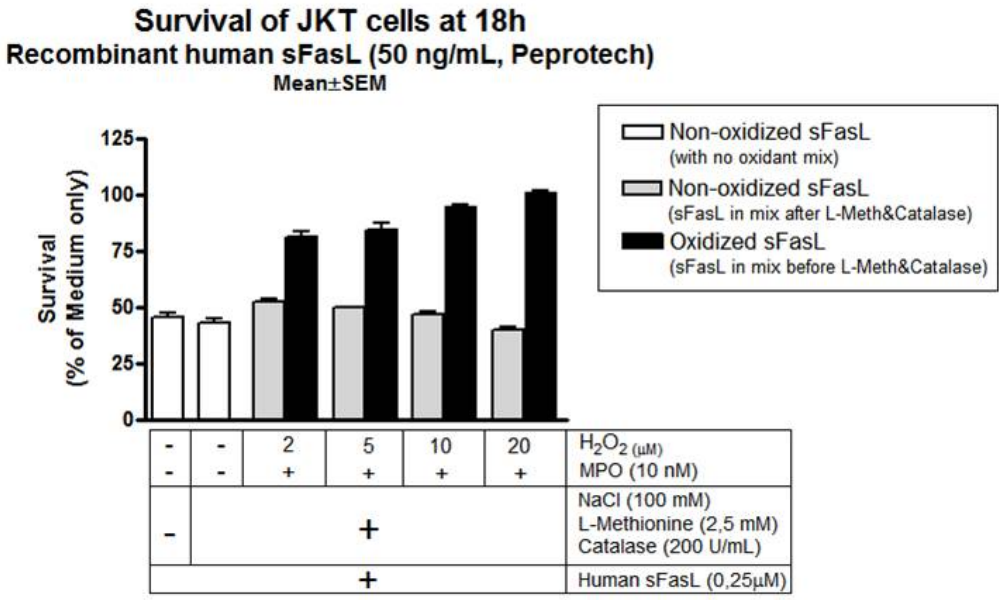


Figure 7.27. Oxidation modulates the biological activity of human sFasL in vitro. We compared the activity of the MPO system-oxidized sFasL to the spontaneously oxidized sFasL containing only two oxidized methionines. Human sFasL was incubated with one dose of MPO (10 nM) and four different concentrations of H_2O_2 (2, 5, 10 or 20 μM) at 37ºC. After 60 min, the oxidative reaction was terminated by adding free L- Methionine and catalase as potent scavengers of HOCl and H_2O_2, respectively. As control, oxidative reaction was terminated by the addition of the oxidant scavengers (free L-methionine and catalase) prior to the addition of the human sFasL protein. Also, sFasL was incubated with the oxidant scavengers but without any oxidant. The samples were added to JKT cells for 18 hours. Cell viability was detected by Alamar Blue assay. The cells treated with spontaneously oxidized sFasL showed a 50% survival whereas the cells treated with the MPO system had higher survival ranging from 75% to 100 % in an H_2O_2 dose dependent manner.

The final effect of oxidation on the activity of a particular protein can be diminished by the presence of other proteins with anti-oxidant properties, such as albumin [644]. Therefore, we next investigated how oxidation affected the activity of human sFasL either in a protein-rich or a protein-free environment. We incubated our KH human sFasL present in the HEK supernatant (protein-rich solution) or an affinity purified KH human sFasL (protein-free solution) with serial doses of HOCl. The activity of KH rh-sFasL in the supernatant tended to increase (although it was not statistically significant) when treated with 10 μM of HOCl, and no changes in the activity were observed with doses of HOCl as high

as 200 μM. As expected, affinity purified rh-sFasL was more susceptible to oxidation. The activity of the purified protein significantly decreased when exposed to very low concentrations of HOCl and it was inhibited with only 0.1 μM of HOCl. In contrast, more than 2000-fold of HOCl was needed to abrogate the activity of the rh-sFasL from HEK supernatant. The activity of rh-sFasL incubated with L-methionine (an HOCl scavenger) prior to the exposure to HOCl had the same activity as the rh-sFasL non-exposed to HOCl. No changes in the level of aggregation were detected by western blot analysis with the doses of HOCl used in these experiments (data not shown). These findings indicate that exposure of sFasL to HOCl results in oxidative changes of the molecule that alter its activity. The results suggest, therefore, that mild oxidation does not decrease the biological activity of human sFasL in a protein-riched environment, and that very high local levels of HOCl might be needed to downregulate or abrogate its biological activity. This may also explain our previous observation that the spontaneously oxidized human sFasL protein, which contained only two oxidized methionines (Met^{15} and Met^{132}), was active and induced cell death in the JKT cells in vitro, whereas further oxidation resulted in a decrease of its biological activity.

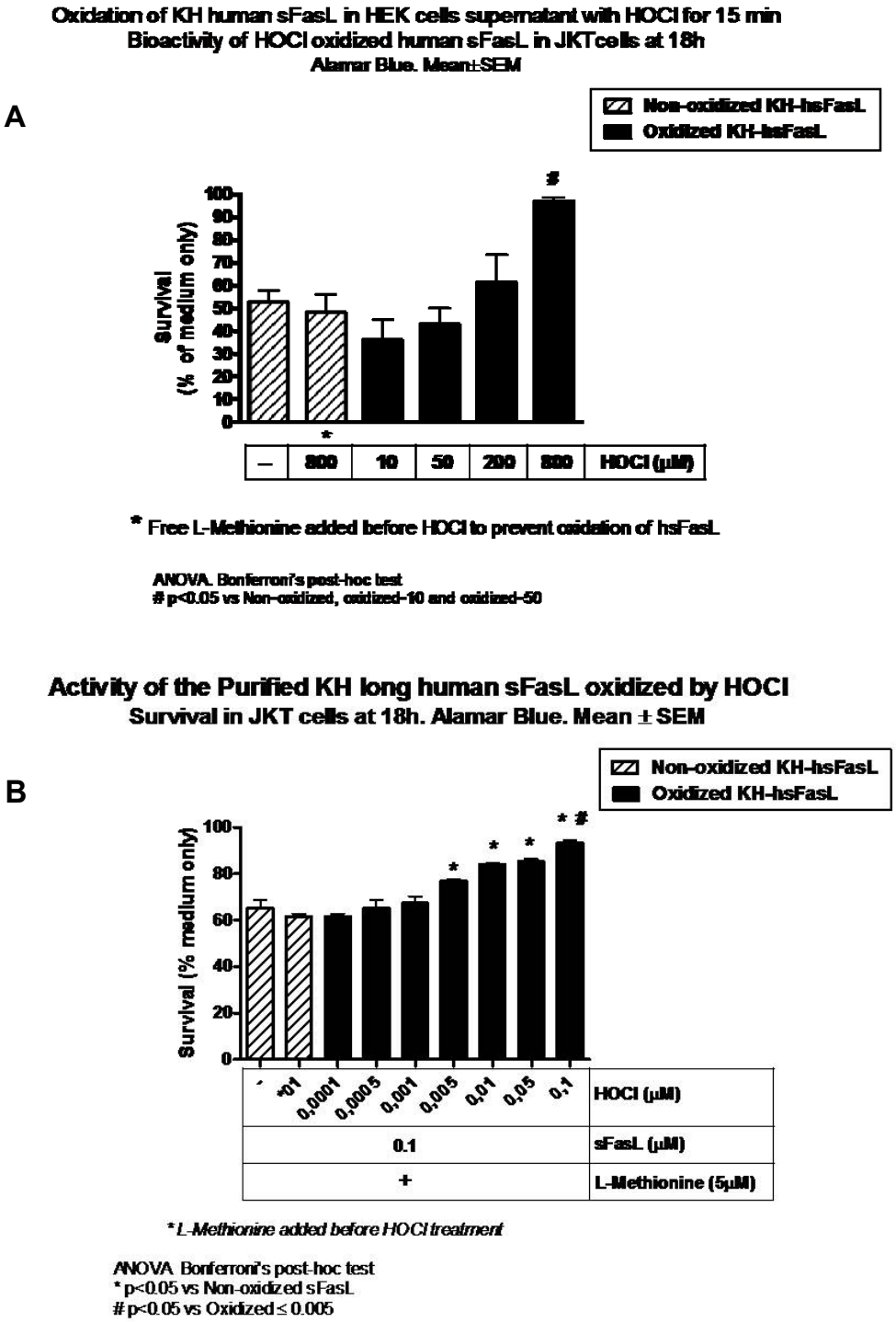


Figure 7.28. The biological activity of human sFasL is preserved under mild oxidation but diminishes under intense levels of oxidation. The long soluble form of human FasL (KH rh-sFas) was cloned and expressed in HEK cells in our laboratory. The KH rh-sFasL secreted to the supernatant of HEK cells was purified by immunoaffinity column. Either the KH rh-sFasL present in the HEK cell supernatant (A) or the purified KH rh-sFasL(B) was exposed to H_2O_2 alone or to serial concentrations of HOCl at 37ºC. After 30 min of incubation, free L-methionie was added to terminate the oxidative reaction. As control, rh-sFasL was previously incubated with L-methionine prior to the addition of HOCl. The rh-sFasL bioactivity in each sample was

then tested in vitro using JKT cells incubated in serum-free media. Cell viability was determined by the Alamar Blue assay after 18 hours of incubation. The JKT cells incubated with media alone was used as negative control. Results represent mean ±SD of three separated experiments performed in duplicate.

7.3.4.5.5. Effects of the reduction of methionine residues on the multimerization status and activity of human sFasL

We have found that oxidation of human sFasL results in the oxidation of methionine residues to methionine sulfoxide, and that it is also associated to the covalent multimerization of human sFasL. To further confirm that methionine oxidation was involved in the covalent mulimerization of human sFasL induced by oxidative conditions, we used dimethyl sulfide (Me_2S) and hydrochloric acid (HCl), which are agents that selectively reduce methionine residues in proteins [630]. Treatment of methionine sulfoxide with Me_2S and high concentrations of HCl results in the rapid conversion of methionine sulfoxide back to methionine. The oxygen exchange between methionine sulfoxide and Me_2S is acid-dependent and the choride anion from HCl is effective in promoting the exchange. The reduction of methionine sulfoxide to methionine is completed only at high concentrations of HCl, because an increased concentration of H_2O in the reaction mix is unfavorable for the reaction to occur. This method does not cause irreversible denaturation of the proteins despite the high concentration of HCl [630]. As shown in Figure 7.29, a 2 hour exposure of human sFasL to a mixture of Me_2S and HCL efficiently dissociated the multimers of human sFasL protein into monomers, strongly supporting that methionine oxidation is the mechanism that originates the covalent multimerization of sFasL.

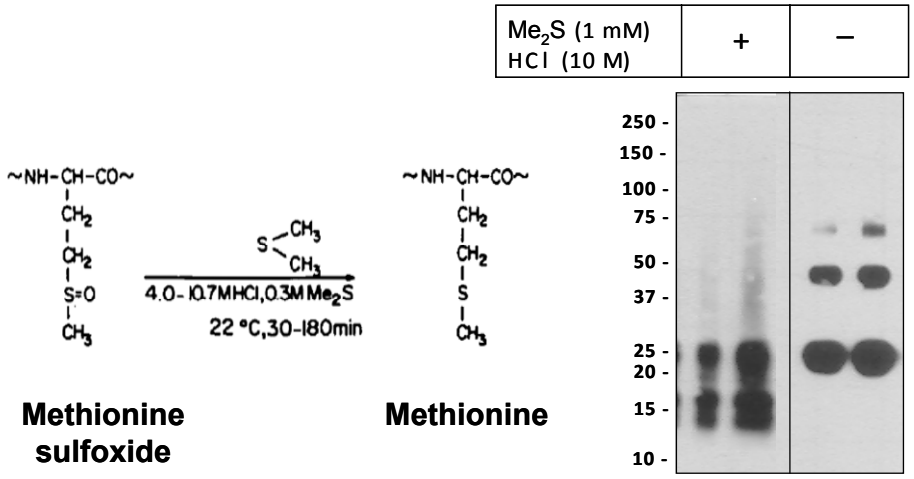


Figure 7.29. Dimethyl sulphide and hydrochloric acid reversed the covalent multimerization of recombinant human sFasL

Recombinant human sFasL (from Peprotech Inc.) was incubated with PBS alone or with dimethyl sulphide (Me_2S) and hydrochloric acid (HCl) at room temperature for 2 hours. After incubation, the samples were filtreated to eliminate Me_2S and HCl and until pH became neutral. The samples were then subjected to electrophoresis in SDS-PAGE gel in non-reduced conditions, and western-blot analysis was performed. Purified human sFasL spontaneously forms covalent multimers (Lane 3, 4). Incubation with Me_2S/HCl complete reversed the covalent multimerization of human sFasL (Lane 1, 2).

To determine whether the oxidation of methionine residues modifies the activity of sFasL, we took advantage of the fact that the commercially available human sFas has two methionine residues that are spontaneously oxidized in basal conditions. We investigated whether the Me_2S/HCl system, which specifically reduces oxidized methionine residues, altered the activity of this form of human sFasL. First, we treated the spontaneously oxidized human sFasL with the Me_2S/HCl system in order to reduce the two methionine sulfoxide residues back to methionine. For this, the rh-sFasL protein was incubated with a mixture of Me_2S and HCl for different periods of time (10, 40 or 120 minutes) as described in Materials and Methods. After incubation, the samples were filtered to eliminate the Me_2S and HCl. The samples were then analyzed by SDS-PAGE electrophoresis under non-reduced conditions, followed by western blotting. The cytotoxic activities of both the spontaneously oxidized and the reduced forms of

human sFasL were also tested in vitro in JKT cells. As shown in Figure 7.30, the reduction of the covalent multimers of human sFasL started after just 10 min of incubation with the Me_2S/HCl system, and this was sufficient to completely inhibit the biological activity of human sFasL. Longer times of incubation were necessary for the Me_2S/HCl to completely dissociate the covalent multimers of human sFasL into monomers.

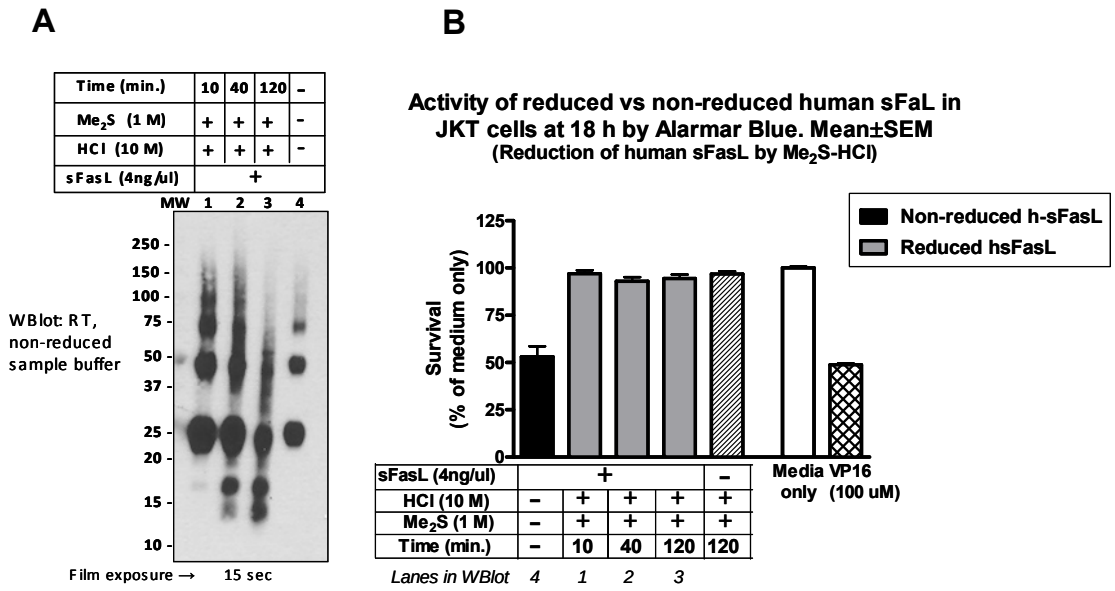


Figure 7.30. Reduction of oxidized methionines reversed the multimerization and reduced the biological activity of recombinant human sFasL.

Recombinant human sFasL (from Peprotech Inc.) was incubated with dimethyl sulphide (Me_2S) and hydrochloric acid (HCl) at room temperature for different periods of time: 10, 40 and 120 min. The samples were filtrated to eliminate the content of Me_2S and HCl, and until the pH became neutral. The samples were then analyzed by electrophoresis in SDS-PAGE gel in non-reduced conditions followed by western blotting (A). The activity of both oxidized and reduced sFasL proteins were also tested in JKT cells (B). Cell viability was measured by the Alamar Blue assay. Purified human sFasL spontaneously forms covalent multimers in aqueous solution (lane 4). Incubation with Me_2S/HCl reversed multimerization of human sFasL in a time-dependent manner (lane 1 to 3). After reduction with Me_2S/HCl, the biological activity of human sFasL significant decreased.

7.3.4.5.6. Effects of the reduction of cysteine residues on the multimerization status and activity of human sFasL

Dithiothreitol (DTT) is widely used to reduce the disulfide bonds between cysteine residues of proteins. Like methionine, cysteine residues also have a sulfhydryl group that is susceptible in solution to both oxidation and disulfide formation [645]. The presence of disulfide bonds in extracellular proteins provides structural strength. To determine whether disulfide bonds between cysteine residues could be involved in the multimerization of human sFasL, we tried to reduce rh-sFasL with high concentrations of DTT without causing protein denaturation. As shown in Figure 7.31, incubation with DTT did not dissociate the multimers of human sFasL, as an equal level of multimerization was present in the rh-sFasL non incubated with DTT. Regarding its biological activity, the rh-sFasL induced a similar percentage of cell death in JKT cells regardless of the presence of DTT in the incubation media. Therefore, incubation with DTT did not decrease the multimerization nor modified the biological activity of human sFasL in vitro. These results suggest that disulfide bonds between cysteine residues are not involved in the covalent multimerization of human sFasL protein promoted by an oxidative environment, and that they do not play a role on the biological activity of sFasL.

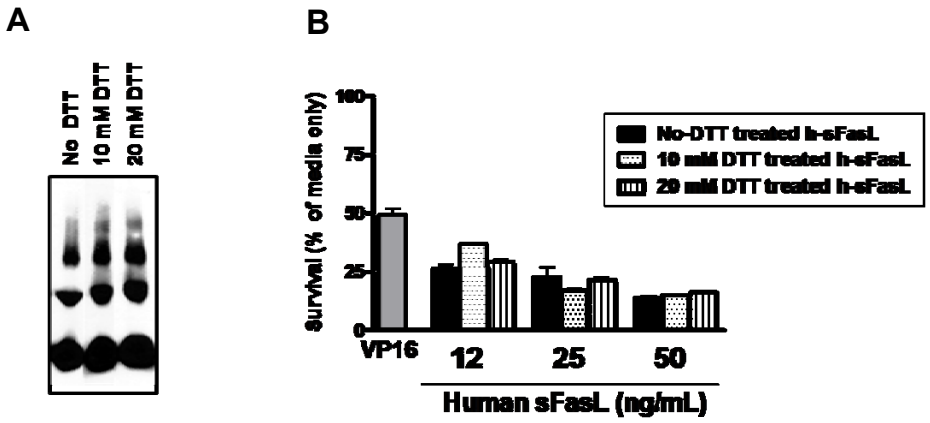


Figure 7.31. Disulfide bonds are not determinants of the multimerization or the control of the biological activity of recombinant human sFasL.

We incubated rh-sFasL with 10 and 20 mM of DTT at 37ºC. One hour later, IAM was added to prevent reformation of the disulfide bonds, and the samples were then filtrated to eliminate the

content of DTT. The samples were then analyzed by electrophoresis in SDS-PAGE gel in non-reduced conditions followed by western blotting (A). The bioactivity of sFasL was also tested in JKT cells (B). Cell viability was measured by the Alamar Blue assay. Incubation with DTT did not reverse the covalent multimerization and did not change the biological activity of rh-sFasL protein. Results represent mean ±SD.

8. – DISCUSSION

The Fas/FasL system has been shown to play a key role in the pathogenesis of ALI/ARDS. The soluble form of FasL (sFasL) is elevated in the BAL fluid of patients who are at-risk for ARDS as well as of those who have established ARDS [467, 468]. However, only the BAL fluid from the later induces apoptosis in human lung distal epithelial cells in vitro [284], suggesting that some factors present in the edema fluid control the biological activity of sFasL. The main goal of the present study was to investigate the structural determinants that control the biological activity of human sFasL. In addition, we studied whether factors present in the BAL fluid of patients with ALI/ARDS modulate the bioactivity of sFasL by changing its molecular conformation. We found that two variants of recombinant human sFasL differing in the presence or absence of the stalk region at the N-terminus (long and short sFasL, respectively) had different biological activities. The long form of human sFasL was also more cytotoxic than its short form in Jurkat cells *in vitro*. Furthermore, only the long form of human sFasL induced lung injury *in vivo* when instilled intratracheally to mice. sFasL-induced lung injury was characterized by local activation of pro-apoptotic pathways, neutrophil infiltration, activation of pro-inflammatory cytokines and increase in alveolar-capillary protein permeability. All these pathological events, however, did not occur or were significantly reduced in Fas-deficient *lpr* mice, indicating that long human sFasL-mediated lung injury was due to the specific activation of Fas receptor. The long form of human sFasL was cleaved when co-incubated with MMP-7 in solution, resulting in a reduction of its activity, most likely explained by the proteolytic processing of the stalk region at its N-terminus. These results indicate that the presence of the stalk region at the N-terminus is a critical determinant of the biological activity of human sFasL in the lungs.

Our study also provided insight into the mechanisms by which the stalk region of sFasL and the pro-inflammatory/oxidative environment in the lungs of patients with ALI/ARDS influences its activity. We showed that sFasL formed trimers and higher molecular weight aggregates that were mediated by ionic forces in basal conditions, and that this degree of aggregation was not affected by the absence of the stalk region. In contrast to basal conditions, we found that the formation of multimers of the endogenous sFasL that accumulates in the alveolar fluid of patients with ARDS was mediated by covalent bonds in addition to ionic forces. Furthermore, exposure to BAL fluid from patients with ARDS increased the level of aggregation of exogenous human sFasL by mechanisms involving oxidation. Exposure to myeloperoxidase-derived oxidants, such as

hypochlorous acid, also increased the aggregation of exogenous sFasL and the stability of such aggregates due to the formation of new intermolecular covalent bonds. The formation of these covalent aggregates of sFasL induced by oxidative stress involved the oxidation of methionine residues, but did not involve the formation of disulfide bonds.

8.1. Relevance of sFasL in ALI

FasL exists in both membrane and soluble forms, both of which can bind and cluster the Fas receptor on the cell membrane of target cells initiating intracellular apoptotic and inflammatory signals [493-495, 625, 646, 647]. Membrane FasL is constitutively expressed in the lung epithelium particularly in the nonciliated, cuboidal airway epithelial cells (Clara cells) and also in the alveolar epithelial type II cells [283, 468, 502]. Soluble FasL (sFasL) can be generated from the cleavage of membrane FasL by the proteolytic action of metalloproteinases (MMPs) [482-488], as well as by the rapid release of pre-formed sFasL in response to cellular stimuli of human monocytes [471, 489-491] and neutrophils [577, 648]. Importantly, the Fas receptor/Fas ligand system is up-regulated in the lungs of patients with ALI and is associated with mortality [284, 468]. In previous studies, it has been shown that sFasL increases in the lung edema fluid of patients with ALI [284, 468] at concentrations that induce apoptosis in human distal lung epithelial cells in vitro [284], suggesting a role of sFasL in the pathogenesis of ALI.

8.1.1. Human sFasL in animal models

In experiments performed in rabbits, the instillation of recombinant human sFasL induced the activation of apoptotic pathways in the alveolar wall and lung injury [549], which suggested that Fas-mediated apoptosis of the alveolar epithelium may be an initial event in the development of some forms of lung injury. In our mouse model, instillation of human sFasL caused structural and functional alterations of the lung that were very similar to those found in patients with ALI. In contrast, the lung damage was significantly attenuated in *lpr* mice, which lack a functional Fas receptor, indicating that sFasL induced lung injury in mouse lungs via the specific activation of the Fas receptor. The results also indicate that the intratracheal instillation of human sFasL in mouse lungs is a good model to study *in vivo* the role of the Fas/FasL system pathway in the development of ALI.

8.1.2. Apoptosis and sFasL-induced lung injury

Intratracheal instillation of human sFasL in the lungs of mice resulted in important structural alterations of the alveolar wall accompanied by an increased permeability to macromolecules, suggesting that both the alveolar endothelial and epithelial barriers were disrupted. This alteration of protein permeability was significantly attenuated in Fas deficient *lpr* mice, indicating that the activation of the Fas/FasL system was required for the disruption of the alveolar epithelial-capillary barrier. The intrinsic mechanisms by which Fas activation alters the alveolar epithelial-capillary barrier, however, are not completely understood. A possibility that has been proposed is that the activation of the Fas/FasL system alters the alveolar epithelial-capillary barrier by inducing apoptosis or dysfunction of alveolar epithelial cells [241, 258, 284, 289, 548, 555]. In this regard, previous studies performed by T.R. Martin's group demonstrated that Fas-mediated lung injury requires the expression of Fas in non-myeloid cells of the lungs such as epithelial cells, and suggested that the alveolar epithelium could be the primary target of Fas-mediated ALI [555]. In chimeric mice only expressing Fas receptor in non-myeloid cells (i.e. epithelial cells) but not in myeloid cells (i.e. PMN and monocytes-macrophages), the instillation of a murine anti-Fas agonistic antibody (Jo-2 Ab) resulted in lung injury associated with neutrophil recruitment and activation of apoptotic pathways, particularly in cells of the alveolar wall [555]. Therefore, one possibility is that Fas ligation results in epithelial and/or endothelial dysfunction via the activation of apoptosis. The exposure of the basement membrane resulting from the denuded alveolar wall may subsequently lead to neutrophil activation, adhesion to endothelial surfaces, and eventual migration into the airspaces. This possibility is supported by the finding that alveolar permeability was increased in mice expressing Fas only in non-myeloid cells, and that these same animals showed significant structural alterations with an increase in the number of apoptotic cells of the alveolar walls [555]. In the model used in the present study, the instillation of human sFasL increased the activity of caspase-3 in the cells of the alveolar wall. Caspase-3 is one of the executioner proteolytic enzymes that cleave proteins needed for cellular function, therefore leading to cell death by apoptosis. In Fas-deficient *lpr* mice, however, the activation of caspase-3 was completely blunted, which was associated with a significant attenuation of the increase in alveolar permeability to macromolecules. Although, we have not determined which cells of the alveolar wall underwent apoptosis in our model, other studies have shown that subsets of the alveolar epithelial cells expressing Fas on their

surface undergo apoptosis in response to Fas ligation [282, 284]. Previous studies by T.R. Martin's group also demonstrated that active sFasL present in the ARDS BAL fluid can induce apoptosis in human distal lung epithelial cells *in vitro* [284]. Therefore, all these findings support the notion that apoptosis mediated by the Fas/FasL system is a mechanism by which the alveolar epithelial barrier is damaged *in vivo* in ALI. This mechanism, however, does not seem to be the only one. Although significant, the increase in apoptosis was limited to a small number of cells in the alveolar wall in all these models, raising the possibility that epithelial cells may lose their barrier function before becoming apoptotic via a mechanism still to be determined.

8.1.3. Inflammation and sFasL-induced lung injury

It has recently been shown that human and mouse lung distal epithelial cells express cytokines upon Fas ligation in vitro, including the expression of potent neutrophil chemoattractants such as IL-8 and KC [539, 566]. These results suggest that activation of the Fas/FasL system not only can induce apoptosis but also cytokine expression in alveolar epithelial cells. The activation of intracellular inflammatory pathways and/or expression of inflammatory mediators in alveolar epithelial cells, therefore, could be a mechanism contributing to the alteration of the alveolar-capillary barrier in Fas-mediated lung injury. The activation of Fas has also been found to induce cytokine release by macrophages and neutrophil recruitment [490, 499, 538], raising the question of whether Fas-mediated injury is due primarily to its effects on the alveolar epithelium or to its effects on the resident alveolar macrophages. In this regard, R.A. Bem and G. Matute-Bello have demonstrated that Fas activation can induce important lung injury and inflammatory responses in mice in which macrophages had been depleted [566]. This supports the concept that the lung damage and the inflammatory responses induced by Fas activation do not depend primarily on resident alveolar macrophages, and may instead depend on cytokine release by alveolar epithelial cells.

The Fas/FasL system-mediated apoptosis and cytokine expression by alveolar epithelial cells could be an initial event in the development of several forms of lung injury. Although PMN may contribute to lung injury in some circumstances, it is not clear whether the first event involves PMN migration, or whether epithelial injury occurs before leukocytes are involved. The primary event leading to lung injury in ARDS has not been established. A prevalent hypothesis is that neutrophils are the primary

players mediating lung injury in ARDS [279, 325, 329, 649]. In this paradigm, uncontrolled neutrophil activation would lead to the accumulation of oxidants and proteases in the lungs, which would cause damage to the neighbouring alveolar cells [330, 650-654]. However, neutrophils can migrate into the lungs of humans or sheeps without causing injury to the epithelial barrier [107, 278, 336]. Blockade of PMN chemoattractants or systemic PMN depletion blocks lung injury in some animal models, but not in all of them.

8.2. Controversies in the biological activity of sFasL

The exact biological activity of sFasL still remains controversial. It has been shown that sFasL can exert both pro- or anti-apoptotic effects, and pro- or anti-inflammatory effects, depending on the system tested [520, 597, 600, 603-606]. Several studies agree that sFasL is less capable of inducing apoptosis and inflammatory responses compared with membrane FasL, and that the proteolytic cleavage of membrane FasL is a mechanism to downregulate its apoptotic effects [482, 597, 598]. Several mechanisms have been suggested to explain the lower cytotoxicity of sFasL compared with membrane FasL. Ligation with sFasL rapidly internalizes Fas, which results in less expression of Fas on the cell surface and makes cells more resistant to membrane FasL-mediated apoptosis [482, 597, 598, 611]. sFasL has also been shown to exert an anti-apoptotic effect in human T cells resulting from the activation of cytoprotective intracellular proteins such as the Bcl-2 anti-apoptotic family members [655, 656]. In addition, cross-linked sFasL has been shown to activate the NF-κB signaling pathway in target cells expressing high levels of FLIP, which inhibited apoptosis and propagated pro-inflammatory responses [514, 528, 533, 657-661]. Even though the capability of sFasL for promoting both apoptotic and inflammatory effects has been demonstrated *in vivo* and *in vitro*, the intrinsic mechanisms by which sFasL initiates inflammatory responses via NF-κB or via other pathways are not completely understood. In animal experiments, intravenous administration of human sFasL induced acute liver failure in mice [662, 663]. Intratracheal instillation of human sFasL in our mouse model led to lung tissue damage associated with the activation of both apoptotic and inflammatory responses. The mechanisms that may explain these discrepancies in the biological activity of sFasL are not completely understood, but proposed mechanisms include differences in the degree of aggregation of the sFasL protein [597, 615, 619], interaction with accessory molecules [612-614], differences in the type of cells studied and in the species of sFasL, and also it may depend on different variants of sFasL [483, 492, 619].

8.2.1. The stalk region is an important determinant for the biological activity of sFasL

Previous studies have shown discrepancies in the activity of the natural cleavage products of FasL. Whereas the naturally cleaved human sFasL did not induce apoptosis in cultured T lymphocytes cells in some studies [485, 597, 603], other lines of evidence indicated that the naturally cleaved sFasL was active and induced apoptosis in epithelial cells *in vivo* and *in vitro* [487, 621]. The extracellular domain of the membrane FasL can be processed by matrix metalloproteinases, such as MMP-3 and MMP-7, at three different cleavage sites, which may create alternative forms of sFasL with different N-terminal sequences [482, 488]. Cleavage at the preferential cleavage sites, located closer to the cell membrane and at the beginning of the stalk region (ELAELR), gives rise to a long form of sFasL containing the TNF homology domain at the C-terminus and most of the stalk region at the N-terminal. Two alterative cleavage sites (SL and ELR) located more distant to the cell membrane give rise to a short form of sFasL containing the TNF-domain but lacking the stalk region. Therefore, we hypothesized in the present study that cleavage at different sites generates specific structural variants that determine the activity of sFasL, therefore explaining the differences in the activity of this soluble protein. We found that structural variants of sFasL had different cytotoxic activity depending on the presence or absence of the stalk region at the N-terminus. Our results showed that the long variant of recombinant human sFasL containing the stalk region induced lung injury in mice, which was characterized by swelling of the alveolar walls, alveolar hemorrhage, neutrophil infiltration and increased alveolar protein permeability. Activation of apoptosis and inflammatory responses appeared to be involved in the development of long human sFasL-mediated injury in the lung, as it induced the activation of apoptotic pathways in cells of the alveolar wall as well as the recruitment of neutrophils and the expression of pro-inflammatory cytokines such as IL-1b, IL-6, KC, MIP-2 and MCP-1 along with a decrease in the anti-inflammatory cytokine IL-4. A short form of human sFasL that lacks the stalk region, however, was not harmful to mouse lungs, and failed to activate pro-apoptotic and pro-inflammatory pathways. Therefore, the absence of the stalk region in the short variant of human sFasL prevented all the tissue damage in mouse lungs *in vivo*. This indicates that the presence of the stalk region at the N-terminus is a critical determinant for the cytotoxic activity of sFasL, which was similar to the lung damage found in patients with ALI/ARDS. The long human sFasL was also more

cytotoxic *in vitro* in our studies, as the exposure of human Jurkat cells to the long variant of human sFasL resulted in significantly higher percentages of cell death than the exposure to the short form of sFasL at all the concentrations tested. To further investigate the relevance of the stalk region, we used MMP-7 to convert the long form of human sFasL to a short form. Incubation of the long variant of human sFasL with MMP-7 resulted in a shift of the sFasL monomers to a lower molecular weight in the Western-blot, and in the appearance of a new band below 10 kDa corresponding to the predictive molecular weight of the stalk region. These structural changes were accompanied by a significant reduction of the cytotoxicity on Jurkat cells compared with the non-treated long human sFasL. These data suggest that MMP-7 reduced the cytotoxicity of the long form of human sFasL *in vitro* by releasing the stalk region at the N-terminus.

8.2.1.1. *The stalk region is not involved in the aggregation of sFasL*

The mechanism by which the presence of the stalk region increases the activity of the sFasL is still unknown. The TNF domain has the structural information for receptor binding and contains a self-association domain, which mediates the aggregation of the sFasL protein. However, soluble trimers of sFasL only composed of the TNF domain do not activate Fas receptor. Each trimeric sFasL binds to three pre-associated molecules of Fas receptor [517], but trimerization of the Fas receptor after Fas ligation is not sufficient to activate intracellular signaling pathways [519, 597, 619]. Several lines of evidence suggest that the initial formation of the trimeric ligand receptor complexes is followed by secondary multimerization into supramolecular clusters of Fas-FasL complexes [519, 622, 625, 664, 665]. The formation of these clusters is associated with the formation of a death-inducing signaling complex (DISC) at the receptor. In the DISC, procaspase-8 is cleaved and released as active caspase-8, which initiates the activation of downstream caspases leading ultimately to apoptosis. These three steps in Fas receptor activation - ligand binding, secondary aggregation of the receptor-ligand complexes and DISC formation - depend on several factors [519]. Proposed factors include the affinity and avidity of FasL-Fas interaction, the manner in which FasL is presented to Fas (as a membrane ligand or as soluble form), and the level of aggregation of FasL.

Cross-linking of sFasL restored the pro-apoptotic activity of sFasL, supporting the idea that aggregation is an important determinant of its activity. It is believed that the lack of apoptotic activity of sFasL observed in some studies was not due to a decrease in binding affinity to Fas, but to an inability to cluster Fas trimers into larger aggregates. In this line, Holler and Berg demonstrated that a cross-linked sFasL retained its capability to aggregate Fas and induce apoptosis [615, 616, 619]. In addition, cross-linked sFasL has also been shown to induce NF-κB nuclear translocation that is responsible for the FasL-induced chemokine production [539]. Therefore, we investigated whether the differences in activity of our two variants of human sFasL were due to different degrees of aggregation. For this, we run both proteins in a non-denaturing gel without SDS, in which protein folding and aggregation are preserved. We found that the long and short variants of human sFasL spontaneously formed trimers and higher molecular weight aggregates in basal conditions. There was no difference in the degree of aggregation between these two variants before or after the purification process. Therefore, the difference in the activity of these two variants of human sFasL cannot be explained by a different degree of aggregation. The self-association domain that has been identified at the beginning of the TNF domain is present in our long and short variants of human sFasL, which may explain the similar ability of both proteins to aggregate regardless of the presence of the stalk region. This also implies that the stalk region is not necessary for aggregation of this protein in solution. The third cleavage-site (ELR), more distant to the cell membrane, is located within the self-associated domain [488], and therefore we cannot discard that other forms of short human sFasL have the ability to aggregate.

8.2.1.2. *Potential mechanisms by which the stalk region influences the biological activity of sFasL*

The stalk region does not participate directly in Fas receptor binding, because the binding sites are located in the TNF domain. However, the stalk region may confer a spatial conformation that increases the affinity of this protein for Fas receptor binding. It could also provide more stability to aggregates of sFasL, for example by stabilizing the TNF domain structure against structural disturbance related to receptor binding. A third possibility is that the presence of the stalk region may promote secondary aggregation of FasL/Fas receptor complexes on the cell surface needed for an efficient activation of intracellular signaling. In contrast to the human sFasL, we found that the

corresponding long and short variants of mouse sFasL formed monomers and dimers, but not trimers. Both variants of mouse sFasL presented a poor cytotoxicity (data no shown). This is in accordance with other studies showing that mouse sFasL is significantly less cytotoxic than human sFasL [619]. Even though previous studies had found that a long form of mouse sFasL comprising the stalk region was able to bind to Fas receptor [620], we were not able to detect activity of our long variant of mouse sFasL in our *in vitro* model using murine and human lymphoma cells bearing Fas receptor on the cell membrane. In agreement with the notion that the degree of aggregation may be an important structural determinant of the activity of sFasL, we propose that the poor activity of the long mouse sFasL in our study may be partly due to its inability to form trimers and larger multimers. Therefore, the presence of the stalk region may not overcome the requirement of the sFasL for aggregation to become properly active.

8.2.1.3. *Cleavage by MMPs may determine the biological activity of sFasL*

Matrix metalloproteinases (MMPs) play a central role in the proteolytic regulation of proteins involved in inflammation and repair, in the turnover of extracellular matrix, and in the pathological destruction of tissue proteins [360, 361, 363-365, 367-369, 666]. The MMP-3 and -7 have been detected in the BAL fluid of patients with ALI/ARDS and both were associated with a worse outcome. MMP-2, MMP-7, MMP-9 and tissue inhibitor of metalloproteinase (TIMP-1) were also elevated in pulmonary epithelium of patients with ALI, asthma and cystic fibrosis [372-374]. However, the specific roles that most MMPs play in lung injury and repair remain largely unknown.

Unlike many MMPs, MMP-7 (also called matrilysin) is constitutively expressed in non-injured, non-inflamed mucosal epithelia in most adult human tissues [389, 390]. In human lungs, MMP-7 is expressed in normal tracheal glands and in tracheal-bronchial epithelium, and can be up-regulated rapidly in the airway epithelium by injury or exposure to bacteria [391, 392]. Some studies also showed that MMP-7 protein expression is up-regulated in injured alveolar epithelium in a variety of human lung diseases, such as ALI, asthma, cystic fibrosis and idiopathic pulmonary fibrosis [372-374]. MMP-7 is an important extracellular protease necessary for epithelial repair [395]. However, it also participates in the progression of lung fibrosis in animal models [384]. This enzyme can be expressed by lung epithelial cells as well as by activated human blood monocytes, but not by differentiated alveolar macrophages [667, 668]. More recently, MMP-7 has been

shown to be released by activated human blood T lymphocytes and bronchoalveolar T lymphocytes [669]. In prostate epithelial cells, MMP-7 can regulate apoptosis by cleaving Fas ligand, which was implicated in prostate involution [487]. In animal models of fibrosis, MMP-7 regulates the expression of KC, an epithelial-derived neutrophil chemotactic factor that promotes inflammation [384, 667, 668, 670]. In this line, an important role of MMP-7 on the transepithelial influx of neutrophils has been reported [384, 670].

The activity of MMP-7 in the lung edema fluid or in the cell membrane of lung epithelial cells, and its potential role in the pathogenesis of ALI are currently unknown. The presence of these MMPs in the airspaces of the lungs of patients with ALI together with the up-regulation of FasL and Fas in the alveolar epithelial cells and in infiltrating inflammatory cells raise the possibility of the cleavage of membrane FasL by MMPs as one of the mechanism by which soluble FasL is released into the airspaces. As explained in previous paragraphs, however, multiple cleavage sites can give rise to different variants of sFasL with different activities.

Our current investigations are aimed to determine which variant of sFasL dominates in the edema fluid of patients with ALI, and whether MMPs released to the airspaces can cleave the sFasL under the oxidative and inflammatory conditions present in the lungs with ALI/ARDS. This is an important question, as the activity of MMP-7 can be modified by the level of oxidation. Mild oxidation of MMP-7 resulted in an increase of its proteolytic activity, whereas moderate and high oxidation resulted in structural changes leading to a decrease of its activity [638, 645]. We have found that incubation of exogenous recombinant human sFasL in the ARDS BAL fluid led to the formation of covalent sFasL multimers in the absence of protein degradation, even though the ARDS BAL fluid is rich in proteases. Interestingly, degradation of human sFasL protein occurred rapidly in the presence of antioxidants (L-Methionine), suggesting that the oxidation of human sFasL may confer resistance to degradation. Nevertheless, further investigations are needed to elucidate this phenomenon.

The data of the present study indicates that sFasL containing the stalk region is capable of inducing tissue injury in the lungs, constituting an operative mechanism by which the Fas/FasL system may mediate lung injury without the requirement for the cell-cell interactions that characterize the membrane FasL. The main limitation of our study is that we tested recombinant forms of human sFasL instead of naturally cleaved

or released sFasL. It is currently unknown which form of sFasL is released to the air spaces of patients with ALI. Releasing different forms of sFasL to the physiological fluid in humans could explain why some patients with increased levels of sFasL in serum did not develop distal organ damage [537, 598, 671]. Different forms of sFasL are probably generated in the lung of these patients, and the net bioactivity of sFasL may depend on which form dominates in their air spaces.

G. Matute-bello and T.R. Martin (mentors) demonstrated that cells present in the BAL fluid of patients with ARDS can release sFasL to the extracellular environment [284]. The majority of the cells in the ARDS BAL fluid are monocytes/macrophages and neutrophils and, therefore, these two types of cells may constitute an important source of sFasL in the airways of patients with ARDS. Pre-formed sFasL has been identified inside these cells [490], which makes possible that part of the sFasL in the BAL fluid was directly released from the cytoplasm and not from the cleavage of membrane FasL. We do not currently know if this pre-formed sFasL has different structural or functional features than the cleavage products of membrane FasL, or if it corresponds to either a long or a short form of sFasL. In prior *in vitro* studies, however, the sFasL naturally released from activated neutrophils induced apoptosis in human lung epithelial cells. It is also possible that the site at which the membrane FasL is cleaved depends on the type of cells in which it is expressed or on the type of MMPs present.

8.2.2. Other mechanisms that may explain the controversies regarding the activity of sFasL

Differences in the type of cells can also explain the differences in the bioactivity of sFasL reported in previous studies. The short human sFasL had no effect in the mouse lungs *in vivo*, but it induced cell death in Jurkat cells *in vitro* (although it was significantly less cytotoxic than the long variant of sFasL). This finding may be explained in part by the different sensitivity of cells to FasL-mediated cell death. The degree of expression, aggregation and internalization of Fas receptor in the cell surface may also determine the sensitivity to sFasL [664, 672-674]. Generally, cells expressing high content of Fas receptor on the surface respond more to Fas ligation [483, 492]. In addition, sFasL has been shown to interact with matrix components such as fibronectin, vitronectin, and collagen IV [613, 614]. Interaction of sFasL with fibronectin restored its cytotoxic activity [613, 614], indicating that binding of sFasL to accessory molecules may

also modulate the activity of this protein. All these are potential explanations for the disagreement between our study and the little cytotoxic effect found in other studies in which forms of human sFasL that did not contained the stalk region were used [619].

8.3. Structure and activity of sFasL in BAL fluid of patients with ALI/ARDS

Prior observations from T.R. Martin's group showed that the concentrations of sFasL in BAL fluid from patients at risk for ARDS and from patients with ARDS were very similar, but only the sFasL present in the ARDS BAL fluid was bioactive [284]. This suggests that cofactors present in the BAL fluid of patients with ARDS potentiate the activity of sFasL. It is likely that the apoptotic effect of sFasL in lung edema fluid depends on complex interactions between agents that promote sFasL aggregation, soluble inhibitors, and intracellular factors. Soluble inhibitors of sFasL are present in the alveolar fluid of patients with ARDS. For example, soluble Fas receptor can be generated from the cleavage of membrane Fas by metalloproteinases, and act as a sink for sFasL [496]. Other potential inhibitor is the Fas decoy receptor DCR3 (TR6), which binds and inactivates sFasL [560]. Additional factors that could modify the effect of the Fas/FasL pathway include the presence of reactive oxygen species [675] and the pre-exposure to inflammatory cytokines such as IL-1β and TNF-α [676].

ALI/ARDS is characterized by a neutrophilic alveolitis and the increased presence of reactive oxygen species (ROS), resulting in the oxidation and/or nitration of intra-alveolar proteins [420, 421, 675, 677, 678]. The air spaces also contain a proteolytic environment with a large content of proteases [679-681]. Because sFasL released to the air spaces of patients with ALI/ARDS may undergo oxidation, we investigated whether oxidants present in the BAL fluid from patients with ALI/ARDS modulate the bioactivity of sFasL by altering the molecular conformation of this protein. Biologically active myeloperoxidase (MPO) and H_2O_2 are detectable in the lung edema fluid of patients with lung injury [675, 682-684]. Hypochlorus acid (HOCl) is synthesized from H_2O_2 and chloride by an MPO-catalyzed reaction, and this MPO-mediated mechanism is the only known source of HOCl in mammals [428]. Activated neutrophils undergo a respiratory burst and produce H_2O_2 which is converted into HOCl by the granule enzyme MPO ($H_2O_2 + Cl^- + H^+ \rightarrow HOCl + H_2O$) [446, 685]. HOCl is one of the major oxidants generated in high concentrations by neutrophils in response to a phagocytic challenge [428]. HOCl is an important component of host defenses against invading organisms, but is also

implicated in neutrophil-mediated tissue damage by causing important alterations of biomolecules including proteins, lipids and nucleic acids [159, 332, 409, 423, 447,]. This oxidant can therefore damage extracellular proteins and inactivate enzymes [159, 332, 409, 416, 423, 425, 442, 443, 675, 686-692]. Reactive oxygen species (ROS) can induce changes in the structural conformation of proteins that ultimately result in modifications of their biological activity *in vivo* [693-695]. ROS force some proteins to adopt new folding structures, promote aggregation and also alter the interaction between different proteins [639, 696-699]. In addition, ROS can facilitate protein degradation [700, 701]. Within the protein, the oxidative reaction can take place in either the peptide bonds or the side-chains of amino acid residues [701-703]. Oxidation of peptide bonds normally is associated to protein cleavage and degradation, whereas oxidation of side-chains of amino acid residues frequently creates covalent cross-links that result in formation of multimers of higher molecular weight. Protein degradation mediated by oxidation is thought to be relevant in protein turn-over [701-703]. The formation of carbonyl groups in oxidized proteins has been used as a marker of damage by oxidative stress in multiple tissues including the lungs [423, 704, 705]. New lines of evidence show that oxidation can also be a mechanism to promote protein activation and may also protect proteins from degradation, as some proteins aggregated by oxidation became more resistant to degradation [634, 706].The fate of the protein after being oxidized mainly depends on the level of ROS and the intensity of oxidation, the intrinsic characteristics of each protein, and the cellular environment.

In basal conditions, human sFasL spontaneously forms trimers and larger multimers that can be reduced to monomers in the presence of SDS, indicating that these multimers are formed by weak intermolecular bonds such as ionic forces. In the present study we found that the BAL fluid from patients with ARDS had multimers of sFasL containing covalent bonds that could not be reduced to monomers in the presence of SDS. This suggested that factors only present in the BAL fluid of patients with ARDS promote stable aggregates of sFasL by forming new covalent bonds in addition to ionic forces. To explore this issue further, we incubated exogenous human sFasL with BAL fluid from normal volunteers, from patients at risk for ARDS, and from patients with established ARDS. Interestingly, only the incubation with BAL fluid from patients with established ARDS led to the formation of covalent multimers of exogenous human sFasL. There are at least two mechanisms that may explain the covalent multimerization of sFasL in BAL fluid of patients with ARDS. First, the lungs of patients with ARDS contain proteins and other molecules that may interact with

sFasL molecules and form stable aggregates. Second, reactive oxygen species in the lungs of patients with ARDS could cause oxidation, nitration or chlorination of sFasL which, in turn, may cause structural alterations and promote multimerization of this protein. Our data strongly support that the oxidation of methionine residues was a key event for the formation of stable covalent multimers of sFasL in our experimental paradigm, and suggest that it could be a mechanism by which sFasL aggregates in the lung edema fluid of patients with ALI/ARDS. In contrast, we did not find evidence of nitration or chlorination in sFasL exposed to reactive oxigen or nitrogen species, indicating that the nitration or chlorination of sFasL were not involved in its multimerization.

We also observed that there was not degradation of sFasL protein despite the fact that BAL fluid from patients with ARDS is a proteolytic environment with high content of metalloproteinases [378] such as MMP-3 and -7 [372-374], which are known to cleave sFasL *in vitro*. In contrast, there was an important and rapid degradation of the exogenous human sFasL when the oxidants present in the ARDS BAL fluid were blocked by the addition of the antioxidant L-methionine. This suggested that the oxidation and covalent aggregation of human sFasL conferred protection against degradation mediated by proteases present in the BAL fluid of patients with ARDS. In general, the aggregation or interaction with other molecules under oxidative conditions can hide the proteolytic recognition site of proteins and, thus, they may become no longer susceptible to degradation by proteases [701, 703, 707]. This could explain in part the resistance of sFasL to degradation in such a proteolytic environment. These data suggest that the oxidation of sFasL present in the air spaces of patients with ARDS leads to its accumulation and to the formation of covalent aggregates, probably resulting in higher stability and resistance to degradation compared with the non-oxidized sFasL aggregates.

There are several potential sources of oxidants in the lungs of patients with ARDS, but our data suggest that the mechanism of sFasL oxidation in ARDS BAL fluid involves MPO-derived HOCl. Some proteins form multimers or aggregates after oxidation with HOCl. Proteins that have been shown to undergo aggregation by HOCl include fibronectin, casein, apolipoprotein A-I from HDL, ovalbumin, α2-macroglobulin and Mn-superoxide dismutase [639, 640, 708-710]. Protein aggregation has been suggested as a pathological mechanism for diseases such as Alzheimer´s, atherosclerosis and

falciform anemia [693, 711-713]. In the present study, we showed that MPO-derived HOCl oxidized and promoted formation of covalent multimers of sFasL *in vitro*. Multimerization of human sFasL was enhanced by MPO-derived HOCl and this was associated with oxidation of methionine residues. These results suggest that MPO-derived oxidants released to the alveolar spaces in injured lungs enhance the multimerization of sFasL, and that such mechanism involves the oxidation of methionine residues.

8.4. HOCl promotes oxidation of methionine residues of sFasL, which results in structural and functional modifications of sFasL

All amino acid residues of proteins are susceptible to oxidation, but the sulfur-containing amino acid residues - i.e. cysteine and methionine - are particularly sensitive to oxidation by most forms of ROS [701, 703]. HOCl modifies proteins in a number of ways, including the formation of chloramines, the conversion of cysteine residues to disulfides, the conversion of methionine into methionine-sulfoxide, the oxidation of tryptophan, the chlorination of tyrosine, and the conversion of amino groups into chloramines [637, 641, 714-717]. Oxidation of these residues induces a variety of conformational changes in proteins that modify their biological activity. Since two cysteine residues are present in human sFasL, we explored the potential role of disulfide bonds between cysteine residues in the multimerization process of sFasL. Unexpectedly, we did not find evidence of oxidation of cysteine residues or of the formation of disulfide bonds between polypeptide chains of human sFasL multimers either in basal or in oxidative conditions. This indicated that the multimerization of sFasL resulting from the exposure to HOCL involved the formation of covalent bonds different from disulfide bridges. Importantly, the human sFasL protein has a high content of methionine residues (8 methionine in the long variant and 7 in the short variant). In the present study, we determined by mass spectrometry that the exposure of human sFasL to oxidants resulted in the specific oxidation of the methionine residues of the protein. The number of methionine residues that were oxidized depended on the intensity of oxidation. Oxidation of all methionine residues by the MPO system occurred with the most intense oxidative conditions used in vitro, and it was not accompanied by changes in any other amino acids. Furthermore, there was no detection of 3-nitrotyrosine or 3-chlorotyrosine in sFasL after exposure to the MPO

system. Together, these findings indicate that methionine is the amino acid most susceptible to oxidation in the human sFasL protein.

8.4.1. Methionine oxidation promotes covalent multimerization of sFasL

To determine whether methionine oxidation was involved in the covalent multimerization of sFasL, we took advantage of the fact that mouse sFasL only contains 2 methionine residues. We expressed mouse sFasL (WT msFasL) and a mutant mouse sFasL generated by site-directed mutagenesis containing 7 methionines in the same position as those in human sFasL (Met-msFasL). Exposure to low doses of HOCl resulted in a higher degree of multimerization of the Met-msFasL protein, which had a higher content of methionine residues. This supports the notion that the oxidation of methionine residues promotes the multimerization of sFasL and facilitates the formation of new covalent forces between molecular units that are very resistant to dissociation. Reduction of the oxidized methionines by dimethyl sulfide (Me_2S) [630] resulted in the dissociation of the mildly-oxidized multimers into monomers, confirming that methionine oxidation was involved in the formation of covalent multimers of sFasL.

8.4.2. Methionine oxidation modulates the biological activity of sFasL

The structural changes mediated by oxidation can lead to modification of the biological activity of proteins [634, 703, 717-720]. Therefore, we investigated whether the level of oxidation and the number of methionine residues oxidized could affect the activity of human sFasL. Surprisingly, the cytotoxicity of human sFasL did not change and even sligthly increased under oxidative conditions. Very intense oxidative conditions were required to significantly decrease or completely inhibit the biological activity of human sFasL *in vitro*. Evidence from mass spectrometry indicated that human sFasL containing only two oxidized methionine residues retained its cytotoxic activity. In contrast, sFasL lost its cytotoxic activity in vitro when all the methionine residues were oxidized. This suggests that the level of oxidation and the number of methionine residues oxidized may determine the biological activity of human sFasL in the lungs.

Methionine is a hydrophobic residue and, unlike other hydrophobic amino acids, its side chain is flexible [634, 635, 721, 722]. Thus, the oxidation of methionine leads to a drastic decrease in hydrophobicity and to the formation of a more rigid structure. Methionine

residues have different susceptibility to oxidation depending on their specific position in the protein [635, 721]. Oxidation of methionine at different locations will have different physicochemical consequences [707]. In this line of thought, methionine residues located near the protein surface are more susceptible to oxidation, but the protein undergoes less conformational changes compared to the oxidation of partially or totally buried methionine residues. The buried methionine residues form hydrophobic clusters with other hydrophobic residues. When buried methionine residues are oxidized, this hydrophobic pocket may open like a pot, exposing the internal residues. This can lead to conformational changes resulting in significant modifications in the biological activity of the protein [636]. The specific mechanisms by which methionine oxidation results in covalent multimerization and functional changes of human sFasL are still unknown, but the oxidation of methionine residues may facilitate the formation of multimers by promoting externalization of its self-association domains. Methionine oxidation not only leads to changes in the structural conformation of the polypeptide backbones but also in the charge pattern and the hydrophobicity on the intermolecular surface, which also determine the affinity of the protein to their targets and, consequently, the activity of the protein [634, 706, 723-725]. Oxidation of methionine residues located in the binding site may also lead either to activation or inhibition of the protein [634, 635]. Because human sFasL with only two oxidized methionine residues (Met^{15} and Met^{132}) retained its cytotoxicity whereas the oxidation of all the methionines was associated with a complete inhibition, it would be interesting to determine which methionine residue/s are implicated in the inactivation of human sFasL. The presence of methionine residues may also have a protective effect against oxidative conditions. In the absence of methionines, high concentrations of oxidants can cleave directly the peptide bonds leading to protein degradation; when the protein contains methionines, these residues are preferentially oxidized potentially preventing the oxidation of peptide bones and other residues.

8.5. Role of neutrophil-derived oxidants in the oxidation of methionine residues of sFasL

Several studies have shown that neutrophils are capable of causing oxidative injury in inflamed lungs. One of the mechanisms of neutrophil-induced damage in the lung is by promoting protein oxidation [408, 423, 726, 727]. Methionine residues are oxidized in proteins of BAL fluid in chronic bronchitis, which is believed to be caused by activated neutrophils migrating into the lung [728]. Activated neutrophils were also capable of

oxidizing methionine residues in vitro in newly synthesized proteins [635]. Moreover, intratracheal instillation of phorbol myristate acetate (PMA), a well-established in vivo model of neutrophil oxidant-mediated ALI, was accompanied by a significant increase in methionine oxidation 4 hours after instillation [727, 729] In another animal model, whole-body exposure to radiation resulted in a significant increase in neutrophil content [727]. The levels of oxidized methionine in the lungs remained unchanged immediately after the irradiation, but they increased significantly in parallel with the influx of neutrophils 2 hours after the irradiation, suggesting that neutrophil-derived oxidants, but not irradiation, caused this methionine oxidation. Although neutrophil influx was reversed within a few hours, the oxidized methionine content remained elevated for at least 24 h, suggesting that the oxidative damage associated with this acute inflammatory response may persist and exacerbate radiation injury [727]. HOCl is one of the most potent oxidants released by activated neutrophils [428]. In an ex-vivo experimental model, perfusion of physiological concentrations of HOCl to the lung effectively oxidized the methionine residues of proteins [726]. In addition, our data showed that HOCl can oxidize methionine residues of sFasL and exert structural and functional modifications of the protein. Altogether, these data suggest that HOCl released by activated neutrophils may be an important mechanism to oxidize and modify the activity of sFasL in the inflamed lung of patients with ARDS.

Oxidation of methionine residues in proteins can be enzymatically reversed by the activity of methionine sulfoxide reductase (Msr), which reduces methionine-sulfoxide (MetO) to its unmodified form (Met) [718, 730, 731]. This enzyme exists in various organisms ranging from bacteria to plants and mammals, being found in human PMN leukocytes and in other tissues [731, 732]. Msr catalyzes the reduction of free and protein-bound methionine-sulfoxide to methionine and constitutes a repair mechanism for oxidatively damaged proteins. Supporting the relevance of methionine oxidation in the modification of the bioactivity of some proteins, it has been proposed that the cyclic oxidation-reduction of methionine residues serves as a built-in oxidant scavenger system to protect such proteins from more extensive irreversible oxidative modification [733, 734].

8.6. Final comments

The sFasL released to the airspaces of patients with ALI/ARDS is active and may damage the alveolar epithelium by mechanism involving apoptosis and inflammation. Therefore, it was important to study the mechanisms that regulate the activity of sFasL in the lung. In addition to its degree of aggregation, the cleavage sites may be an important determinant of the activity of this protein, as variants of sFasL with different N-terminal sequences may be generated. We found that the presence of the stalk region at the N-terminus determined the biological activity of human sFasL in the lung. Variants of human sFasL containing the stalk region were more active than variants lacking the stalk region. It is possible that the net biological activity of sFasL in the airspaces depends on the ratio of the different structural variants of sFasL present. The generation of reactive oxygen species by activated neutrophils in the lungs may lead to structural and functional modifications of sFasL. In this oxidative environment, human sFasL retains its biological activity and forms stable aggregates probably by mechanisms involving oxidation of methionine residues. It is likely that a certain level of oxidation favors the activity of sFasL, as well as prevents its degradation or inhibition by other factors present in the lung of patients with ALI/ARDS. We cannot discard, however, that the activity of sFasL may be progressively reduced as the local level of oxidants increases in the injured lung. The presence of metalloproteinases may also be a factor that controls the activity of sFasL in the lung of these patients, but no information has been reported yet in this regard. We consider that targeting the stalk region at the N-terminal may be a potential mechanism to modify the biological activity of sFasL *in vivo*.

Our future lines of investigation will address the effect of the neutrophil-derived oxidants on the structure and the activity of sFasL in vivo in an inflamed lung. From our study, we know that sFasL forms covalent aggregates only in the ARDS BAL fluid probably by mechanisms involving oxidation of methionine residues. However, the real oxidation status of the endogenous sFasL in the air spaces of these patients is still unknown. It is likely that the degree of oxidation of sFasL varies during the different phases of the development of ALI, which could be associated with different biological activity. The high content of methionine residues of human sFasL makes this protein very sensitive to oxidation, but this protein can be oxidized while retaining its cytotoxicity. This is in accordance with previous studies showing that sFasL was

present in the BAL fluid of patients with ARDS in an active form despite the presence of oxidants and proteases that have been shown to alter and destroy other proteins in the lung [284]. Under oxidative conditions, the formation of covalent multimers of sFasL may be a protective mechanism not only to maintain its activity but also to prevent its degradation. We cannot discard, however, that the formation of covalent aggregates mediated by oxidation may also prevent the interaction of sFasL with inhibitory factors such as soluble Fas receptor and DRc3 present in the lung edema fluid of patients with lung injury, which have been shown to inhibit sFasL. On the other hand, oxidation may facilitate the interaction of sFasL with factors that help restore its cytotoxicity, such as the extracellular matrix component fibronectin [613].

8.7. Summary of results

The major findings of this study are summarized as follows:

1. Soluble Fas Ligand (sFasL) can be detected by Western-blotting in the BAL fluid of patients with ARDS, where it tends to aggregate forming at least dimers. No sFasL is detected by Western-blotting in the BAL fluid from patients at-risk for ARDS or from normal volunteers.

2. The intratracheal instillation of human sFasL in mice induces severe lung injury, which is characterized by important alterations of the structure of alveolar walls, increased alveolar-capillary protein permeability, local activation of pro-apoptotic pathways, neutrophil infiltration and induction of expression of pro-inflammatory cytokines.

Human sFasL-induced lung injury occurs via the specific activation of Fas receptor, as the intratracheal instillation of human sFasL in *lpr* mice harboring an inactive Fas receptor does not induce lung damage.

3. The presence of the stalk region at the N-terminus is a determinant of the biological activity of diverse variants of sFasL. Importantly:

a. In vivo, only the intratracheal instillation of a long variant of human sFasL containing the stalk region induces lung injury in mice *in vivo*, whereas a short form of human sFasL lacking the stalk region does not.
b. In vitro, the incubation of human Jurkat cells with the long form of human sFasL leads to a higher percentage of cell death compared with its short form.
c. Both the long and the short forms of human sFasL aggregate spontaneously into trimers and larger molecular weight aggregates under basal conditions, which are mediated by ionic forces in vitro.
d. The co-incubation of the long form of sFasL with MMP-7 results in the cleavage of sFasL and in the reduction of its biological activity in vitro.

4. The endogenous sFasL that accumulates in the BAL fluid of patients with ARDS forms multimers that are mediated by both ionic and covalent forces.

5. An oxidative environment, such as that characteristically present in the airspaces of patients with ALI/ARDS, modifies the structure and activity of human sFasL via a mechanism that involves its methionine residues, which is supported by the following findings:

a. Only the BAL fluid from patients with ARDS, but not from patients at-risk for ARDS or normal volunteers, promotes the aggregation and increases the activity of exogenous human sFasL. Despite the high content in proteases of ARDS BAL fluid, the exogenous human sFasL is not degraded in co-incubation, unless the oxidants in the ARDS BAL fluid are neutralized by adding L-Methionine, a potent HOCl scavenger.

b. The exposure to oxidative agents, but not to nitrosative agents, enhances the multimerization of recombinant human sFasL. Free L-Methionine, a potent HOCl scavenger, prevents the multimerization of sFasL under oxidative conditions.

c. The introduction by site-directed mutagenesis of additional methionine residues into mouse sFasL (which has only 2 methionines compared with 7 methionines in the human sFasL) significantly enhances its multimerization under mild oxidative conditions. Such. multimers of sFasL can not be dissociated under reducing conditions indicating the formation of covalent bonds between the monomers.

d. Exposure to MPO-derived oxidants results in the specific oxidation of the methionine residues in sFasL as assessed by mass spectrometry.

e. Aggregates of sFasL containing oxidized methionines are only dissociated to monomers by dimethyl sulphide (Me_2O) and hydrochloric acid (HCl) but not by dithiothreitol (DTT) indicating that methionine oxidation but not the formation of disulfide bonds is involved in the multimerization of sFasL under oxidative conditions. The dissociation of sFasL multimers with Me_2O results in a decrease of its biological activity in vitro.

f. Purified human sFasL, without the presence of other proteins, contains two oxidized methionine residues, indicating that this protein is very susceptible to oxidation. The biological activity of human sFasL is preserved and even increases slightly under mild oxidative conditions. An intense level of oxidation is required to diminish the biological activity of sFasL.

9. – CONCLUSIONS

The results of the present study support the following major conclusions:

1. The soluble form of Fas Ligand (sFasL) induces lung injury in mouse lungs via the specific activation of the Fas receptor.

2. The stalk region at the N-terminus is a critical determinant of the biological activity of human sFasL in the lungs in vivo and in vitro, but it does not affect the degree of aggregation of the protein. The stalk region of sFasL can be cleaved in vitro by MMP-7, leading to a reduction of its biological activity.

3. The BAL fluid from patients with ARDS contains factors that:
   a. Increase the activity of sFasL
   b. Enhance its aggregation by mechanisms involving the formation of new covalent inter-molecular bonds, which confer more stability and resistance to degradation.

4. The methionine residues of sFasL are critical determinants of the function of this protein under oxidative conditions. In particular, the exposure to a pro-oxidative environment modifies the bioactivity of sFasL and promotes its aggregation by mechanisms involving the specific oxidation of the methionine residues of the protein.

Globally, the results from the study suggest that:

a. The biological activity of human sFasL in the lung edema fluid of patients with ALI/ARDS may be determined by the ratio of structural variants of sFasL containing or not containing the stalk region. MMP-7 and other metalloproteinases released to the airspaces of injured lungs may be important endogenous modulators of the activity of sFasL by inducing its cleavage at different sites.

b. Neutrophil-derived oxidants present in the airspaces of ARDS patients may induce multimerization and modify the bioactivity of sFasL in the lungs of these patients.

c. The stalk region of sFasL may be a valuable therapeutic target for modifying the biological activity of this protein in vivo.

FUTURE DIRECTIONS

Our studies suggest an important role for sFasL in the pathogenesis of lung injury in humans and animals. Our future directions will include studies to determine whether sFasL may cause acute and chronic epithelial injury in the lungs. We propose to create novel transgenic mice with inducible over-expression of human sFasL in the lungs to test the hypothesis that its release into the airspaces produces an inflammatory phenotype that leads to acute lung injury and long term fibrosis. In addition, we will perform experiments to determine the mechanisms by which the stalk region determines the activity of the sFasL *in vitro* and *in vivo*. We will evaluate whether the stalk region increases the affinity of sFasL for receptor binding and/or facilitates the aggregation of Fas receptor on the cell surface of target cells needed for the transmission of intracellular signals. We will also explore other mechanisms such as accessory molecules or proteases that may modify the activity of sFasL in the injured lungs. The better knowledge of these molecular mechanisms may help us to initiate strategies to modify the biological activity of sFasL in vivo. This could include the use of small molecules to target the stalk region at the onset of the development of lung diseases in which sFasL is involved, such as ALI and IPF. In the long run, improving our understanding of the biology of the Fas/FasL system will enhance the understanding of the basic pathophysiology of ALI and ARDS, and offer new opportunities for the treatment of patients with lung injury.

10. – BIBLIOGRAPHY

1. Ashbaugh DG, Bigelow DB, Petty TL, Levine BE. Acute respiratory distress in adults. Lancet 1967;2:319-23.
2. Petty TL, Ashbaugh DG. The adult respiratory distress syndrome. Clinical features, factors influencing prognosis and principles of management. Chest 1971;60:233-9.
3. Murray JF, Matthay MA, Luce JM, Flick MR. An expanded definition of the adult respiratory distress syndrome. Am Rev Respir Dis 1988;138:720-3.
4. Doyle RL, Szaflarski N, Modin GW, Wiener-Kronish JP, Matthay MA. Identification of patients with acute lung injury. Predictors of mortality. Am J Respir Crit Care Med 1995;152:1818-24.
5. Zilberberg MD, Epstein SK. Acute lung injury in the medical ICU: comorbid conditions, age, etiology, and hospital outcome. Am J Respir Crit Care Med 1998;157:1159-64.
6. Heffner JE, Brown LK, Barbieri CA, Harpel KS, DeLeo J. Prospective validation of an acute respiratory distress syndrome predictive score. Am J Respir Crit Care Med 1995;152:1518-26.
7. Bernard GR, Artigas A, Brigham KL, Carlet J, Falke K, Hudson L, Lamy M, Legall JR, Morris A, Spragg R. The American-European Consensus Conference on ARDS. Definitions, mechanisms, relevant outcomes, and clinical trial coordination. Am J Respir Crit Care Med 1994;149:818-24.
8. Bernard GR, Artigas A, Brigham KL, Carlet J, Falke K, Hudson L, Lamy M, LeGall JR, Morris A, Spragg R. Report of the American-European Consensus conference on acute respiratory distress syndrome: definitions, mechanisms, relevant outcomes, and clinical trial coordination. Consensus Committee. J Crit Care 1994;9:72-81.
9. Sloane PJ, Gee MH, Gottlieb JE, Albertine KH, Peters SP, Burns JR, Machiedo G, Fish JE. A multicenter registry of patients with acute respiratory distress syndrome. Physiology and outcome. Am Rev Respir Dis 1992;146:419-26.
10. Monchi M, Bellenfant F, Cariou A, Joly LM, Thebert D, Laurent I, Dhainaut JF, Brunet F. Early predictive factors of survival in the acute respiratory distress syndrome. A multivariate analysis. Am J Respir Crit Care Med 1998;158:1076-81.
11. Abraham E, Matthay MA, Dinarello CA, Vincent JL, Cohen J, Opal SM, Glauser M, Parsons P, Fisher CJ, Jr., Repine JE. Consensus conference definitions for sepsis, septic shock, acute lung injury, and acute respiratory distress syndrome: time for a reevaluation. Crit Care Med 2000;28:232-5.
12. Rubenfeld GD, Caldwell E, Granton J, Hudson LD, Matthay MA. Interobserver variability in applying a radiographic definition for ARDS. Chest 1999;116:1347-53.
13. Meade MO, Cook RJ, Guyatt GH, Groll R, Kachura JR, Bedard M, Cook DJ, Slutsky AS, Stewart TE. Interobserver variation in interpreting chest radiographs for the diagnosis of acute respiratory distress syndrome. Am J Respir Crit Care Med 2000;161:85-90.
14. Ferguson ND, Kacmarek RM, Chiche JD, Singh JM, Hallett DC, Mehta S, Stewart TE. Screening of ARDS patients using standardized ventilator settings: influence on enrollment in a clinical trial. Intensive Care Med 2004;30:1111-6.

15. Gowda MS, Klocke RA. Variability of indices of hypoxemia in adult respiratory distress syndrome. Crit Care Med 1997;25:41-5.
16. Esteban A, Fernandez-Segoviano P, Frutos-Vivar F, Aramburu JA, Najera L, Ferguson ND, Alia I, Gordo F, Rios F. Comparison of clinical criteria for the acute respiratory distress syndrome with autopsy findings. Ann Intern Med 2004;141:440-5.
17. Luce JM. The imperfect diagnosis of acute respiratory distress syndrome. Crit Care Med 2005;33:2419-20.
18. Ware LB. Pathophysiology of acute lung injury and the acute respiratory distress syndrome. Semin Respir Crit Care Med 2006;27:337-49.
19. Frutos-Vivar F, Ferguson ND, Esteban A. Epidemiology of acute lung injury and acute respiratory distress syndrome. Semin Respir Crit Care Med 2006;27:327-36.
20. Conference report: Mechanisms of acute respiratory failure. Am Rev Respir Dis 1977;115:1071-8.
21. Frutos-Vivar F, Nin N, Esteban A. Epidemiology of acute lung injury and acute respiratory distress syndrome. Curr Opin Crit Care 2004;10:1-6.
22. Webster NR, Cohen AT, Nunn JF. Adult respiratory distress syndrome--how many cases in the UK? Anaesthesia 1988;43:923-6.
23. Villar J, Slutsky AS. The incidence of the adult respiratory distress syndrome. Am Rev Respir Dis 1989;140:814-6.
24. Thomsen GE, Morris AH. Incidence of the adult respiratory distress syndrome in the state of Utah. Am J Respir Crit Care Med 1995;152:965-71.
25. Luhr OR, Antonsen K, Karlsson M, Aardal S, Thorsteinsson A, Frostell CG, Bonde J. Incidence and mortality after acute respiratory failure and acute respiratory distress syndrome in Sweden, Denmark, and Iceland. The ARF Study Group. Am J Respir Crit Care Med 1999;159:1849-61.
26. Arroliga AC, Ghamra ZW, Perez Trepichio A, Perez Trepichio P, Komara JJ, Jr., Smith A, Wiedemann HP. Incidence of ARDS in an adult population of northeast Ohio. Chest 2002;121:1972-6.
27. Bersten AD, Edibam C, Hunt T, Moran J. Incidence and mortality of acute lung injury and the acute respiratory distress syndrome in three Australian States. Am J Respir Crit Care Med 2002;165:443-8.
28. Brun-Buisson C, Minelli C, Bertolini G, Brazzi L, Pimentel J, Lewandowski K, Bion J, Romand JA, Villar J, Thorsteinsson A, Damas P, Armaganidis A, Lemaire F. Epidemiology and outcome of acute lung injury in European intensive care units. Results from the ALIVE study. Intensive Care Med 2004;30:51-61.
29. Matthay MA, Zimmerman GA, Esmon C, Bhattacharya J, Coller B, Doerschuk CM, Floros J, Gimbrone MA, Jr., Hoffman E, Hubmayr RD, Leppert M, Matalon S, Munford R, Parsons P, Slutsky AS, Tracey KJ, Ward P, Gail DB, Harabin AL. Future research directions in acute lung injury: summary of a National Heart, Lung, and Blood Institute working group. Am J Respir Crit Care Med 2003;167:1027-35.
30. Milberg JA, Davis DR, Steinberg KP, Hudson LD. Improved survival of patients with acute respiratory distress syndrome (ARDS): 1983-1993. JAMA 1995;273:306-9.

31. Suchyta MR, Clemmer TP, Orme JF, Jr., Morris AH, Elliott CG. Increased survival of ARDS patients with severe hypoxemia (ECMO criteria). Chest 1991;99:951-5.
32. Montgomery AB, Stager MA, Carrico CJ, Hudson LD. Causes of mortality in patients with the adult respiratory distress syndrome. Am Rev Respir Dis 1985;132:485-9.
33. Ventilation with lower tidal volumes as compared with traditional tidal volumes for acute lung injury and the acute respiratory distress syndrome. The Acute Respiratory Distress Syndrome Network. N Engl J Med 2000;342:1301-8.
34. Zambon M, Vincent JL. Mortality rates for patients with acute lung injury/ARDS have decreased over time. Chest 2008;133:1120-7.
35. Davidson TA, Caldwell ES, Curtis JR, Hudson LD, Steinberg KP. Reduced quality of life in survivors of acute respiratory distress syndrome compared with critically ill control patients. JAMA 1999;281:354-60.
36. Abel SJ, Finney SJ, Brett SJ, Keogh BF, Morgan CJ, Evans TW. Reduced mortality in association with the acute respiratory distress syndrome (ARDS). Thorax 1998;53:292-4.
37. Rubenfeld GD, Caldwell E, Peabody E, Weaver J, Martin DP, Neff M, Stern EJ, Hudson LD. Incidence and outcomes of acute lung injury. N Engl J Med 2005;353:1685-93.
38. Ware LB, Matthay MA. The acute respiratory distress syndrome. N Engl J Med 2000;342:1334-49.
39. Iscimen R, Cartin-Ceba R, Yilmaz M, Khan H, Hubmayr RD, Afessa B, Gajic O. Risk factors for the development of acute lung injury in patients with septic shock: an observational cohort study. Crit Care Med 2008;36:1518-22.
40. Pepe PE, Potkin RT, Reus DH, Hudson LD, Carrico CJ. Clinical predictors of the adult respiratory distress syndrome. Am J Surg 1982;144:124-30.
41. Fowler AA, Hamman RF, Good JT, Benson KN, Baird M, Eberle DJ, Petty TL, Hyers TM. Adult respiratory distress syndrome: risk with common predispositions. Ann Intern Med 1983;98:593-7.
42. Bux J, Sachs UJ. The pathogenesis of transfusion-related acute lung injury (TRALI). Br J Haematol 2007;136:788-99.
43. Dicker RA, Morabito DJ, Pittet JF, Campbell AR, Mackersie RC. Acute respiratory distress syndrome criteria in trauma patients: why the definitions do not work. J Trauma 2004;57:522-6; discussion 526-8.
44. Croce MA, Tolley EA, Claridge JA, Fabian TC. Transfusions result in pulmonary morbidity and death after a moderate degree of injury. J Trauma 2005;59:19-23; discussion 23-4.
45. Jawa RS, Anillo S, Kulaylat MN. Transfusion-related acute lung injury. J Intensive Care Med 2008;23:109-21.
46. Ma YF, Sheng L, Gu J, Zhang M, Jiang GY. Analysis of clinical risk factors associated with mortality of severely injured multiple trauma patients with acute lung injury. Chin Med J (Engl) 2009;122:701-5.
47. Silliman CC. Transfusion-related acute lung injury. Transfus Med Rev 1999;13:177-86.
48. Rubenfeld GD, Herridge MS. Epidemiology and outcomes of acute lung injury. Chest 2007;131:554-62.

49. Bone RC, Francis PB, Pierce AK. Intravascular coagulation associated with the adult respiratory distress syndrome. Am J Med 1976;61:585-9.
50. Jia X, Malhotra A, Saeed M, Mark RG, Talmor D. Risk factors for ARDS in patients receiving mechanical ventilation for > 48 h. Chest 2008;133:853-61.
51. Hudson LD, Milberg JA, Anardi D, Maunder RJ. Clinical risks for development of the acute respiratory distress syndrome. Am J Respir Crit Care Med 1995;151:293-301.
52. Eberhard LW, Morabito DJ, Matthay MA, Mackersie RC, Campbell AR, Marks JD, Alonso JA, Pittet JF. Initial severity of metabolic acidosis predicts the development of acute lung injury in severely traumatized patients. Crit Care Med 2000;28:125-31.
53. Moss M, Bucher B, Moore FA, Moore EE, Parsons PE. The role of chronic alcohol abuse in the development of acute respiratory distress syndrome in adults. JAMA 1996;275:50-4.
54. Iribarren C, Jacobs DR, Jr., Sidney S, Gross MD, Eisner MD. Cigarette smoking, alcohol consumption, and risk of ARDS: a 15-year cohort study in a managed care setting. Chest 2000;117:163-8.
55. TenHoor T, Mannino DM, Moss M. Risk factors for ARDS in the United States: analysis of the 1993 National Mortality Followback Study. Chest 2001;119:1179-84.
56. Pelosi P, D'Onofrio D, Chiumello D, Paolo S, Chiara G, Capelozzi VL, Barbas CS, Chiaranda M, Gattinoni L. Pulmonary and extrapulmonary acute respiratory distress syndrome are different. Eur Respir J Suppl 2003;42:48s-56s.
57. Gattinoni L, Pelosi P, Suter PM, Pedoto A, Vercesi P, Lissoni A. Acute respiratory distress syndrome caused by pulmonary and extrapulmonary disease. Different syndromes? Am J Respir Crit Care Med 1998;158:3-11.
58. Rocco PR, Pelosi P. Pulmonary and extrapulmonary acute respiratory distress syndrome: myth or reality? Curr Opin Crit Care 2008;14:50-5.
59. Zilberberg MD, Stern LS, Wiederkehr DP, Doyle JJ, Shorr AF. Anemia, transfusions and hospital outcomes among critically ill patients on prolonged acute mechanical ventilation: a retrospective cohort study. Crit Care 2008;12:R60.
60. Eisner MD, Thompson T, Hudson LD, Luce JM, Hayden D, Schoenfeld D, Matthay MA, Acute Respiratory Distress Syndrome N. Efficacy of low tidal volume ventilation in patients with different clinical risk factors for acute lung injury and the acute respiratory distress syndrome. Am J Respir Crit Care Med 2001;164:231-6.
61. Headley AS, Tolley E, Meduri GU. Infections and the inflammatory response in acute respiratory distress syndrome. Chest 1997;111:1306-21.
62. Looney MR. Newly recognized causes of acute lung injury: transfusion of blood products, severe acute respiratory syndrome, and avian influenza. Clin Chest Med 2006;27:591-600; abstract viii.
63. Brower RG, Fessler HE. Mechanical ventilation in acute lung injury and acute respiratory distress syndrome. Clin Chest Med 2000;21:491-510, viii.
64. Gibot S, Cariou A, Drouet L, Rossignol M, Ripoll L. Association between a genomic polymorphism within the CD14 locus and septic shock susceptibility and mortality rate. Crit Care Med 2002;30:969-73.

65. Cariou A, Chiche JD, Charpentier J, Dhainaut JF, Mira JP. The era of genomics: impact on sepsis clinical trial design. Crit Care Med 2002;30:S341-8.
66. Reiner AP, Wurfel MM, Lange LA, Carlson CS, Nord AS, Carty CL, Rieder MJ, Desmarais C, Jenny NS, Iribarren C, Walston JD, Williams OD, Nickerson DA, Jarvik GP. Polymorphisms of the IL1-receptor antagonist gene (IL1RN) are associated with multiple markers of systemic inflammation. Arterioscler Thromb Vasc Biol 2008;28:1407-12.
67. Wurfel MM, Gordon AC, Holden TD, Radella F, Strout J, Kajikawa O, Ruzinski JT, Rona G, Black RA, Stratton S, Jarvik GP, Hajjar AM, Nickerson DA, Rieder M, Sevransky J, Maloney JP, Moss M, Martin G, Shanholtz C, Garcia JG, Gao L, Brower R, Barnes KC, Walley KR, Russell JA, Martin TR. Toll-like receptor 1 polymorphisms affect innate immune responses and outcomes in sepsis. Am J Respir Crit Care Med 2008;178:710-20.
68. Marshall RP, Webb S, Bellingan GJ, Montgomery HE, Chaudhari B, McAnulty RJ, Humphries SE, Hill MR, Laurent GJ. Angiotensin converting enzyme insertion/deletion polymorphism is associated with susceptibility and outcome in acute respiratory distress syndrome. Am J Respir Crit Care Med 2002;166:646-50.
69. Marshall RP, Webb S, Hill MR, Humphries SE, Laurent GJ. Genetic polymorphisms associated with susceptibility and outcome in ARDS. Chest 2002;121:68S-69S.
70. Schwartz DB, Bone RC, Balk RA, Szidon JP. Hepatic dysfunction in the adult respiratory distress syndrome. Chest 1989;95:871-5.
71. Bernard GR, Sopko G, Cerra F, Demling R, Edmunds H, Kaplan S, Kessler L, Masur H, Parsons P, Shure D, Webb C, Weidemann H, Weinmann G, Williams D. Pulmonary artery catheterization and clinical outcomes: National Heart, Lung, and Blood Institute and Food and Drug Administration Workshop Report. Consensus Statement. JAMA 2000;283:2568-72.
72. Donati SY, Papazian L. Role of open-lung biopsy in acute respiratory distress syndrome. Curr Opin Crit Care 2008;14:75-9.
73. Kao KC, Tsai YH, Wu YK, Chen NH, Hsieh MJ, Huang SF, Huang CC. Open lung biopsy in early-stage acute respiratory distress syndrome. Crit Care 2006;10:R106.
74. Terminella L, Sharma G. Diagnostic studies in patients with acute respiratory distress syndrome. Semin Thorac Cardiovasc Surg 2006;18:2-7.
75. Avecillas JF, Freire AX, Arroliga AC. Clinical epidemiology of acute lung injury and acute respiratory distress syndrome: incidence, diagnosis, and outcomes. Clin Chest Med 2006;27:549-57; abstract vii.
76. Papazian L, Doddoli C, Chetaille B, Gernez Y, Thirion X, Roch A, Donati Y, Bonnety M, Zandotti C, Thomas P. A contributive result of open-lung biopsy improves survival in acute respiratory distress syndrome patients. Crit Care Med 2007;35:755-62.
77. Zaccardelli DS, Pattishall EN. Clinical diagnostic criteria of the adult respiratory distress syndrome in the intensive care unit. Crit Care Med 1996;24:247-51.
78. Bulpa PA, Dive AM, Mertens L, Delos MA, Jamart J, Evrard PA, Gonzalez MR, Installe EJ. Combined bronchoalveolar lavage and transbronchial lung biopsy: safety and yield in ventilated patients. Eur Respir J 2003;21:489-94.

79. Aberle DR, Brown K. Radiologic considerations in the adult respiratory distress syndrome. Clin Chest Med 1990;11:737-54.
80. Goodman LR, Fumagalli R, Tagliabue P, Tagliabue M, Ferrario M, Gattinoni L, Pesenti A. Adult respiratory distress syndrome due to pulmonary and extrapulmonary causes: CT, clinical, and functional correlations. Radiology 1999;213:545-52.
81. Gattinoni L, Bombino M, Pelosi P, Lissoni A, Pesenti A, Fumagalli R, Tagliabue M. Lung structure and function in different stages of severe adult respiratory distress syndrome. JAMA 1994;271:1772-9.
82. Pittet JF, Mackersie RC, Martin TR, Matthay MA. Biological markers of acute lung injury: prognostic and pathogenetic significance. Am J Respir Crit Care Med 1997;155:1187-205.
83. Chesnutt AN, Matthay MA, Tibayan FA, Clark JG. Early detection of type III procollagen peptide in acute lung injury. Pathogenetic and prognostic significance. Am J Respir Crit Care Med 1997;156:840-5.
84. Clark JG, Milberg JA, Steinberg KP, Hudson LD. Type III procollagen peptide in the adult respiratory distress syndrome. Association of increased peptide levels in bronchoalveolar lavage fluid with increased risk for death. Ann Intern Med 1995;122:17-23.
85. Bachofen M, Weibel ER. Structural alterations of lung parenchyma in the adult respiratory distress syndrome. Clin Chest Med 1982;3:35-56.
86. Heresi GA, Arroliga AC, Wiedemann HP, Matthay MA. Pulmonary artery catheter and fluid management in acute lung injury and the acute respiratory distress syndrome. Clin Chest Med 2006;27:627-35; abstract ix.
87. Gropper MA, Wiener-Kronish J. The epithelium in acute lung injury/acute respiratory distress syndrome. Curr Opin Crit Care 2008;14:11-5.
88. Berthiaume Y, Matthay MA. Alveolar edema fluid clearance and acute lung injury. Respir Physiol Neurobiol 2007;159:350-9.
89. Guidot DM, Folkesson HG, Jain L, Sznajder JI, Pittet JF, Matthay MA. Integrating acute lung injury and regulation of alveolar fluid clearance. Am J Physiol Lung Cell Mol Physiol 2006;291:L301-6.
90. Sartori C, Matthay MA. Alveolar epithelial fluid transport in acute lung injury: new insights. Eur Respir J 2002;20:1299-313.
91. Zemans RL, Matthay MA. Bench-to-bedside review: the role of the alveolar epithelium in the resolution of pulmonary edema in acute lung injury. Crit Care 2004;8:469-77.
92. Ware LB, Matthay MA. Alveolar fluid clearance is impaired in the majority of patients with acute lung injury and the acute respiratory distress syndrome. Am J Respir Crit Care Med 2001;163:1376-83.
93. Hastings RH, Folkesson HG, Matthay MA. Mechanisms of alveolar protein clearance in the intact lung. Am J Physiol Lung Cell Mol Physiol 2004;286:L679-89.
94. Folkesson HG, Matthay MA, Westrom BR, Kim KJ, Karlsson BW, Hastings RH. Alveolar epithelial clearance of protein. J Appl Physiol 1996;80:1431-45.
95. Matute-Bello G, Liles WC, Radella F, 2nd, Steinberg KP, Ruzinski JT, Hudson LD, Martin TR. Modulation of neutrophil apoptosis by granulocyte colony-stimulating factor and granulocyte/macrophage colony-stimulating factor during the course of acute respiratory distress syndrome. Crit Care Med 2000;28:1-7.

96. Matute-Bello G, Liles WC, Radella F, 2nd, Steinberg KP, Ruzinski JT, Jonas M, Chi EY, Hudson LD, Martin TR. Neutrophil apoptosis in the acute respiratory distress syndrome. Am J Respir Crit Care Med 1997;156:1969-77.
97. Herridge MS, Cheung AM, Tansey CM, Matte-Martyn A, Diaz-Granados N, Al-Saidi F, Cooper AB, Guest CB, Mazer CD, Mehta S, Stewart TE, Barr A, Cook D, Slutsky AS, Canadian Critical Care Trials G. One-year outcomes in survivors of the acute respiratory distress syndrome. N Engl J Med 2003;348:683-93.
98. McHugh LG, Milberg JA, Whitcomb ME, Schoene RB, Maunder RJ, Hudson LD. Recovery of function in survivors of the acute respiratory distress syndrome. Am J Respir Crit Care Med 1994;150:90-4.
99. Aberle DR, Wiener-Kronish JP, Webb WR, Matthay MA. Hydrostatic versus increased permeability pulmonary edema: diagnosis based on radiographic criteria in critically ill patients. Radiology 1988;168:73-9.
100. Ware LB, Fang X, Matthay MA. Protein C and thrombomodulin in human acute lung injury. Am J Physiol Lung Cell Mol Physiol 2003;285:L514-21.
101. Matthay MA, Ware LB. Plasma protein C levels in patients with acute lung injury: prognostic significance. Crit Care Med 2004;32:S229-32.
102. Anderson WR, Thielen K. Correlative study of adult respiratory distress syndrome by light, scanning, and transmission electron microscopy. Ultrastruct Pathol 1992;16:615-28.
103. Frank JA, Briot R, Lee JW, Ishizaka A, Uchida T, Matthay MA. Physiological and biochemical markers of alveolar epithelial barrier dysfunction in perfused human lungs. Am J Physiol Lung Cell Mol Physiol 2007;293:L52-9.
104. Matthay MA, Flori HR, Conner ER, Ware LB. Alveolar epithelial fluid transport: basic mechanisms and clinical relevance. Proc Assoc Am Physicians 1998;110:496-505.
105. Matthay MA, Fukuda N, Frank J, Kallet R, Daniel B, Sakuma T. Alveolar epithelial barrier. Role in lung fluid balance in clinical lung injury. Clin Chest Med 2000;21:477-90.
106. Matthay MA, Robriquet L, Fang X. Alveolar epithelium: role in lung fluid balance and acute lung injury. Proc Am Thorac Soc 2005;2:206-13.
107. Wiener-Kronish JP, Albertine KH, Matthay MA. Differential responses of the endothelial and epithelial barriers of the lung in sheep to Escherichia coli endotoxin. J Clin Invest 1991;88:864-75.
108. Holter JF, Weiland JE, Pacht ER, Gadek JE, Davis WB. Protein permeability in the adult respiratory distress syndrome. Loss of size selectivity of the alveolar epithelium. J Clin Invest 1986;78:1513-22.
109. Pratt PC, Vollmer RT, Shelburne JD, Crapo JD. Pulmonary morphology in a multihospital collaborative extracorporeal membrane oxygenation project. I. Light microscopy. Am J Pathol 1979;95:191-214.
110. Bachofen M, Weibel ER. Alterations of the gas exchange apparatus in adult respiratory insufficiency associated with septicemia. Am Rev Respir Dis 1977;116:589-615.
111. Mondschein MS. Pathophysiology of pulmonary fibrosis occurring in association with the adult respiratory distress syndrome. Mt Sinai J Med 1979;46:79-84.
112. Bitterman PB. Pathogenesis of fibrosis in acute lung injury. Am J Med 1992;92:39S-43S.

113. Zapol WM, Trelstad RL, Coffey JW, Tsai I, Salvador RA. Pulmonary fibrosis in severe acute respiratory failure. Am Rev Respir Dis 1979;119:547-54.
114. Tomashefski JF, Jr. Pulmonary pathology of acute respiratory distress syndrome. Clin Chest Med 2000;21:435-66.
115. Saint S, Matthay MA. Risk reduction in the intensive care unit. Am J Med 1998;105:515-23.
116. Heyland DK. Enteral and parenteral nutrition in the seriously ill, hospitalized patient: a critical review of the evidence. J Nutr Health Aging 2000;4:31-41.
117. Martindale RG, McClave SA, Vanek VW, McCarthy M, Roberts P, Taylor B, Ochoa JB, Napolitano L, Cresci G, American College of Critical Care M, Directors ASPENBo. Guidelines for the provision and assessment of nutrition support therapy in the adult critically ill patient: Society of Critical Care Medicine and American Society for Parenteral and Enteral Nutrition: Executive Summary. Crit Care Med 2009;37:1757-61.
118. McClave SA, Martindale RG, Vanek VW, McCarthy M, Roberts P, Taylor B, Ochoa JB, Napolitano L, Cresci G, the ASPENBoD, the American College of Critical Care M. Guidelines for the Provision and Assessment of Nutrition Support Therapy in the Adult Critically Ill Patient:: Society of Critical Care Medicine (SCCM) and American Society for Parenteral and Enteral Nutrition (A.S.P.E.N.). JPEN J Parenter Enteral Nutr 2009;33:277-316.
119. Elamin EM, Camporesi E. Evidence-based nutritional support in the intensive care unit. Int Anesthesiol Clin 2009;47:121-38.
120. Heyland D, Cook DJ, Winder B, Brylowski L, Van deMark H, Guyatt G. Enteral nutrition in the critically ill patient: a prospective survey. Crit Care Med 1995;23:1055-60.
121. Simmons RS, Berdine GG, Seidenfeld JJ, Prihoda TJ, Harris GD, Smith JD, Gilbert TJ, Mota E, Johanson WG, Jr. Fluid balance and the adult respiratory distress syndrome. Am Rev Respir Dis 1987;135:924-9.
122. Humphrey H, Hall J, Sznajder I, Silverstein M, Wood L. Improved survival in ARDS patients associated with a reduction in pulmonary capillary wedge pressure. Chest 1990;97:1176-80.
123. Mitchell JP, Schuller D, Calandrino FS, Schuster DP. Improved outcome based on fluid management in critically ill patients requiring pulmonary artery catheterization. Am Rev Respir Dis 1992;145:990-8.
124. National Heart L, Blood Institute Acute Respiratory Distress Syndrome Clinical Trials N, Wiedemann HP, Wheeler AP, Bernard GR, Thompson BT, Hayden D, deBoisblanc B, Connors AF, Jr., Hite RD, Harabin AL. Comparison of two fluid-management strategies in acute lung injury. N Engl J Med 2006;354:2564-75.
125. Wiedemann HP, Tai DY. Adult respiratory distress syndrome (ARDS): current management, future directions. Cleve Clin J Med 1997;64:365-72.
126. National Heart L, Blood Institute Acute Respiratory Distress Syndrome Clinical Trials N, Wheeler AP, Bernard GR, Thompson BT, Schoenfeld D, Wiedemann HP, deBoisblanc B, Connors AF, Jr., Hite RD, Harabin AL. Pulmonary-artery versus central venous catheter to guide treatment of acute lung injury. N Engl J Med 2006;354:2213-24.
127. Marty AT, Prather J, Matloff JM, Schauble J. Oncotic effects of dilutional bypass, albumin, and diuretics. Arch Surg 1973;107:21-5.

128. Ware LB, Matthay MA. Clinical practice. Acute pulmonary edema. N Engl J Med 2005;353:2788-96.
129. Park WY, Goodman RB, Steinberg KP, Ruzinski JT, Radella F, 2nd, Park DR, Pugin J, Skerrett SJ, Hudson LD, Martin TR. Cytokine balance in the lungs of patients with acute respiratory distress syndrome. Am J Respir Crit Care Med 2001;164:1896-903.
130. Webb HH, Tierney DF. Experimental pulmonary edema due to intermittent positive pressure ventilation with high inflation pressures. Protection by positive end-expiratory pressure. Am Rev Respir Dis 1974;110:556-65.
131. Dreyfuss D, Basset G, Soler P, Saumon G. Intermittent positive-pressure hyperventilation with high inflation pressures produces pulmonary microvascular injury in rats. Am Rev Respir Dis 1985;132:880-4.
132. Dreyfuss D, Soler P, Basset G, Saumon G. High inflation pressure pulmonary edema. Respective effects of high airway pressure, high tidal volume, and positive end-expiratory pressure. Am Rev Respir Dis 1988;137:1159-64.
133. Parker JC, Townsley MI, Rippe B, Taylor AE, Thigpen J. Increased microvascular permeability in dog lungs due to high peak airway pressures. J Appl Physiol 1984;57:1809-16.
134. Corbridge TC, Wood LD, Crawford GP, Chudoba MJ, Yanos J, Sznajder JI. Adverse effects of large tidal volume and low PEEP in canine acid aspiration. Am Rev Respir Dis 1990;142:311-5.
135. Slutsky AS, Tremblay LN. Multiple system organ failure. Is mechanical ventilation a contributing factor? Am J Respir Crit Care Med 1998;157:1721-5.
136. Plotz FB, Vreugdenhil HA, van Vught AJ, Heijnen CJ. Mechanical ventilation and multiple organ failure. Lancet 2003;361:1654.
137. Nin N, Penuelas O, de Paula M, Lorente JA, Fernandez-Segoviano P, Esteban A. Ventilation-induced lung injury in rats is associated with organ injury and systemic inflammation that is attenuated by dexamethasone. Crit Care Med 2006;34:1093-8.
138. Ranieri VM, Suter PM, Tortorella C, De Tullio R, Dayer JM, Brienza A, Bruno F, Slutsky AS. Effect of mechanical ventilation on inflammatory mediators in patients with acute respiratory distress syndrome: a randomized controlled trial. JAMA 1999;282:54-61.
139. Santos CC, Zhang H, Liu M, Slutsky AS. Bench-to-bedside review: Biotrauma and modulation of the innate immune response. Crit Care 2005;9:280-6.
140. Tremblay LN, Slutsky AS. Ventilator-induced injury: from barotrauma to biotrauma. Proc Assoc Am Physicians 1998;110:482-8.
141. Uhlig S, Ranieri M, Slutsky AS. Biotrauma hypothesis of ventilator-induced lung injury. Am J Respir Crit Care Med 2004;169:314-5; author reply 315.
142. Dreyfuss D, Saumon G. Ventilator-induced lung injury: lessons from experimental studies. Am J Respir Crit Care Med 1998;157:294-323.
143. Petrucci N, Iacovelli W. Ventilation with lower tidal volumes versus traditional tidal volumes in adults for acute lung injury and acute respiratory distress syndrome. Cochrane Database Syst Rev 2003:CD003844.
144. Bouvet F, Dreyfuss D, Lebtahi R, Martet G, Le Guludec D, Saumon G. Noninvasive evaluation of acute capillary permeability changes during high-volume ventilation in rats with and without hypercapnic acidosis. Crit Care Med 2005;33:155-60; discussion 250-2.

145. Parsons PE, Eisner MD, Thompson BT, Matthay MA, Ancukiewicz M, Bernard GR, Wheeler AP, Network NARDSCT. Lower tidal volume ventilation and plasma cytokine markers of inflammation in patients with acute lung injury. Crit Care Med 2005;33:1-6; discussion 230-2.
146. Brower RG, Lanken PN, MacIntyre N, Matthay MA, Morris A, Ancukiewicz M, Schoenfeld D, Thompson BT, National Heart L, Blood Institute ACTN. Higher versus lower positive end-expiratory pressures in patients with the acute respiratory distress syndrome. N Engl J Med 2004;351:327-36.
147. Cheng IW, Eisner MD, Thompson BT, Ware LB, Matthay MA, Acute Respiratory Distress Syndrome N. Acute effects of tidal volume strategy on hemodynamics, fluid balance, and sedation in acute lung injury. Crit Care Med 2005;33:63-70; discussion 239-40.
148. Antonelli M, Conti G, Moro ML, Esquinas A, Gonzalez-Diaz G, Confalonieri M, Pelaia P, Principi T, Gregoretti C, Beltrame F, Pennisi MA, Arcangeli A, Proietti R, Passariello M, Meduri GU. Predictors of failure of noninvasive positive pressure ventilation in patients with acute hypoxemic respiratory failure: a multi-center study. Intensive Care Med 2001;27:1718-28.
149. Rossaint R, Gerlach H, Schmidt-Ruhnke H, Pappert D, Lewandowski K, Steudel W, Falke K. Efficacy of inhaled nitric oxide in patients with severe ARDS. Chest 1995;107:1107-15.
150. Dellinger RP, Zimmerman JL, Taylor RW, Straube RC, Hauser DL, Criner GJ, Davis K, Jr., Hyers TM, Papadakos P. Effects of inhaled nitric oxide in patients with acute respiratory distress syndrome: results of a randomized phase II trial. Inhaled Nitric Oxide in ARDS Study Group. Crit Care Med 1998;26:15-23.
151. Taylor RW, Zimmerman JL, Dellinger RP, Straube RC, Criner GJ, Davis K, Jr., Kelly KM, Smith TC, Small RJ, Inhaled Nitric Oxide in ASG. Low-dose inhaled nitric oxide in patients with acute lung injury: a randomized controlled trial. JAMA 2004;291:1603-9.
152. Anzueto A, Baughman RP, Guntupalli KK, Weg JG, Wiedemann HP, Raventos AA, Lemaire F, Long W, Zaccardelli DS, Pattishall EN. Aerosolized surfactant in adults with sepsis-induced acute respiratory distress syndrome. Exosurf Acute Respiratory Distress Syndrome Sepsis Study Group. N Engl J Med 1996;334:1417-21.
153. Long W, Thompson T, Sundell H, Schumacher R, Volberg F, Guthrie R. Effects of two rescue doses of a synthetic surfactant on mortality rate and survival without bronchopulmonary dysplasia in 700- to 1350-gram infants with respiratory distress syndrome. The American Exosurf Neonatal Study Group I. J Pediatr 1991;118:595-605.
154. Lasnier JM, Wangensteen OD, Schmitz LS, Gross CR, Ingbar DH. Terbutaline stimulates alveolar fluid resorption in hyperoxic lung injury. J Appl Physiol 1996;81:1723-9.
155. Azzam ZS, Saldias FJ, Comellas A, Ridge KM, Rutschman DH, Sznajder JI. Catecholamines increase lung edema clearance in rats with increased left atrial pressure. J Appl Physiol 2001;90:1088-94.
156. Matthay MA, Folkesson HG, Verkman AS. Salt and water transport across alveolar and distal airway epithelia in the adult lung. Am J Physiol 1996;270:L487-503.

157. Matalon S, Benos DJ, Jackson RM. Biophysical and molecular properties of amiloride-inhibitable Na+ channels in alveolar epithelial cells. Am J Physiol 1996;271:L1-22.
158. Yue G, Russell WJ, Benos DJ, Jackson RM, Olman MA, Matalon S. Increased expression and activity of sodium channels in alveolar type II cells of hyperoxic rats. Proc Natl Acad Sci U S A 1995;92:8418-22.
159. Matthay MA, Geiser T, Matalon S, Ischiropoulos H. Oxidant-mediated lung injury in the acute respiratory distress syndrome. Crit Care Med 1999;27:2028-30.
160. Jiang X, Ingbar DH, O'Grady SM. Adrenergic stimulation of Na+ transport across alveolar epithelial cells involves activation of apical Cl- channels. Am J Physiol 1998;275:C1610-20.
161. Jiang X, Ingbar DH, O'Grady SM. Adrenergic regulation of ion transport across adult alveolar epithelial cells: effects on Cl- channel activation and transport function in cultures with an apical air interface. J Membr Biol 2001;181:195-204.
162. Matthay MA, Abraham E. Beta-adrenergic agonist therapy as a potential treatment for acute lung injury. Am J Respir Crit Care Med 2006;173:254-5.
163. Perkins GD, McAuley DF, Thickett DR, Gao F. The beta-agonist lung injury trial (BALTI): a randomized placebo-controlled clinical trial. Am J Respir Crit Care Med 2006;173:281-7.
164. Bernard GR, Luce JM, Sprung CL, Rinaldo JE, Tate RM, Sibbald WJ, Kariman K, Higgins S, Bradley R, Metz CA, et al. High-dose corticosteroids in patients with the adult respiratory distress syndrome. N Engl J Med 1987;317:1565-70.
165. Luce JM, Montgomery AB, Marks JD, Turner J, Metz CA, Murray JF. Ineffectiveness of high-dose methylprednisolone in preventing parenchymal lung injury and improving mortality in patients with septic shock. Am Rev Respir Dis 1988;138:62-8.
166. Meduri GU, Marik PE, Annane D. Prolonged glucocorticoid treatment in acute respiratory distress syndrome: Evidence supporting effectiveness and safety. Crit Care Med 2009;37:1800-3.
167. Bernard GR, Wheeler AP, Russell JA, Schein R, Summer WR, Steinberg KP, Fulkerson WJ, Wright PE, Christman BW, Dupont WD, Higgins SB, Swindell BB. The effects of ibuprofen on the physiology and survival of patients with sepsis. The Ibuprofen in Sepsis Study Group. N Engl J Med 1997;336:912-8.
168. Bernard GR, Vincent JL, Laterre PF, LaRosa SP, Dhainaut JF, Lopez-Rodriguez A, Steingrub JS, Garber GE, Helterbrand JD, Ely EW, Fisher CJ, Jr., Recombinant human protein CWEiSSsg. Efficacy and safety of recombinant human activated protein C for severe sepsis. N Engl J Med 2001;344:699-709.
169. Liu KD, Levitt J, Zhuo H, Kallet RH, Brady S, Steingrub J, Tidswell M, Siegel MD, Soto G, Peterson MW, Chesnutt MS, Phillips C, Weinacker A, Thompson BT, Eisner MD, Matthay MA. Randomized clinical trial of activated protein C for the treatment of acute lung injury. Am J Respir Crit Care Med 2008;178:618-23.
170. Laterre PF, Wittebole X, Dhainaut JF. Anticoagulant therapy in acute lung injury. Crit Care Med 2003;31:S329-36.
171. Cross CE, Frei B, Louie S. The adult respiratory distress syndrome (ARDS) and oxidative stress: therapeutic implications. Adv Exp Med Biol 1990;264:435-48.

172. Ketoconazole for early treatment of acute lung injury and acute respiratory distress syndrome: a randomized controlled trial. The ARDS Network. JAMA 2000;283:1995-2002.
173. Panos RJ, Bak PM, Simonet WS, Rubin JS, Smith LJ. Intratracheal instillation of keratinocyte growth factor decreases hyperoxia-induced mortality in rats. J Clin Invest 1995;96:2026-33.
174. Yano T, Deterding RR, Simonet WS, Shannon JM, Mason RJ. Keratinocyte growth factor reduces lung damage due to acid instillation in rats. Am J Respir Cell Mol Biol 1996;15:433-42.
175. Yano T, Mason RJ, Pan T, Deterding RR, Nielsen LD, Shannon JM. KGF regulates pulmonary epithelial proliferation and surfactant protein gene expression in adult rat lung. Am J Physiol Lung Cell Mol Physiol 2000;279:L1146-58.
176. Wang Y, Folkesson HG, Jayr C, Ware LB, Matthay MA. Alveolar epithelial fluid transport can be simultaneously upregulated by both KGF and beta-agonist therapy. J Appl Physiol 1999;87:1852-60.
177. Waters CM, Savla U, Panos RJ. KGF prevents hydrogen peroxide-induced increases in airway epithelial cell permeability. Am J Physiol 1997;272:L681-9.
178. Gillis P, Savla U, Volpert OV, Jimenez B, Waters CM, Panos RJ, Bouck NP. Keratinocyte growth factor induces angiogenesis and protects endothelial barrier function. J Cell Sci 1999;112 (Pt 12):2049-57.
179. Desachy A, Gissot V, et le groupe A. [Gas embolism during protective ventilation for acute respiratory distress syndrome]. Ann Fr Anesth Reanim 2006;25:299-301.
180. Chastre J, Trouillet JL, Vuagnat A, Joly-Guillou ML, Clavier H, Dombret MC, Gibert C. Nosocomial pneumonia in patients with acute respiratory distress syndrome. Am J Respir Crit Care Med 1998;157:1165-72.
181. Niederman MS, Fein AM. Sepsis syndrome, the adult respiratory distress syndrome, and nosocomial pneumonia. A common clinical sequence. Clin Chest Med 1990;11:633-56.
182. Bhatia M, Moochhala S. Role of inflammatory mediators in the pathophysiology of acute respiratory distress syndrome. J Pathol 2004;202:145-56.
183. Gullo A, Berlot G, Viviani M. The role of adult respiratory distress syndrome in the multiple organ dysfunction syndrome. Acta Anaesthesiol Scand Suppl 1996;109:70-3.
184. Khadaroo RG, Marshall JC. ARDS and the multiple organ dysfunction syndrome. Common mechanisms of a common systemic process. Crit Care Clin 2002;18:127-41.
185. Hudson LD, Hough CL. Therapy for late-phase acute respiratory distress syndrome. Clin Chest Med 2006;27:671-7; abstract ix-x.
186. Hirvela ER. Advances in the management of acute respiratory distress syndrome: protective ventilation. Arch Surg 2000;135:126-35.
187. Kramer N, Meyer TJ, Meharg J, Cece RD, Hill NS. Randomized, prospective trial of noninvasive positive pressure ventilation in acute respiratory failure. Am J Respir Crit Care Med 1995;151:1799-806.
188. Nuckton TJ, Alonso JA, Kallet RH, Daniel BM, Pittet JF, Eisner MD, Matthay MA. Pulmonary dead-space fraction as a risk factor for death in the acute respiratory distress syndrome. N Engl J Med 2002;346:1281-6.

189. Valta P, Uusaro A, Nunes S, Ruokonen E, Takala J. Acute respiratory distress syndrome: frequency, clinical course, and costs of care. Crit Care Med 1999;27:2367-74.
190. Aggarwal AN, Gupta D, Behera D, Jindal SK. Analysis of static pulmonary mechanics helps to identify functional defects in survivors of acute respiratory distress syndrome. Crit Care Med 2000;28:3480-3.
191. Ghio AJ, Elliott CG, Crapo RO, Berlin SL, Jensen RL. Impairment after adult respiratory distress syndrome. An evaluation based on American Thoracic Society recommendations. Am Rev Respir Dis 1989;139:1158-62.
192. Elliott CG, Rasmusson BY, Crapo RO, Morris AH, Jensen RL. Prediction of pulmonary function abnormalities after adult respiratory distress syndrome (ARDS). Am Rev Respir Dis 1987;135:634-8.
193. Agarwal R, Aggarwal AN, Gupta D, Behera D, Jindal SK. Etiology and outcomes of pulmonary and extrapulmonary acute lung injury/ARDS in a respiratory ICU in North India. Chest 2006;130:724-9.
194. Suchyta MR, Elliott CG, Jensen RL, Crapo RO. Predicting the presence of pulmonary function impairment in adult respiratory distress syndrome survivors. Respiration 1993;60:103-8.
195. Hopkins RO, Weaver LK, Pope D, Orme JF, Bigler ED, Larson LV. Neuropsychological sequelae and impaired health status in survivors of severe acute respiratory distress syndrome. Am J Respir Crit Care Med 1999;160:50-6.
196. Weinert CR, Gross CR, Kangas JR, Bury CL, Marinelli WA. Health-related quality of life after acute lung injury. Am J Respir Crit Care Med 1997;156:1120-8.
197. Wong HR. ARDS. The future. Crit Care Clin 2002;18:177-96.
198. Bowler RP, Duda B, Chan ED, Enghild JJ, Ware LB, Matthay MA, Duncan MW. Proteomic analysis of pulmonary edema fluid and plasma in patients with acute lung injury. Am J Physiol Lung Cell Mol Physiol 2004;286:L1095-104.
199. Chang DW, Hayashi S, Gharib SA, Vaisar T, King ST, Tsuchiya M, Ruzinski JT, Park DR, Matute-Bello G, Wurfel MM, Bumgarner R, Heinecke JW, Martin TR. Proteomic and computational analysis of bronchoalveolar proteins during the course of the acute respiratory distress syndrome. Am J Respir Crit Care Med 2008;178:701-9.
200. Pereira RF, Halford KW, O'Hara MD, Leeper DB, Sokolov BP, Pollard MD, Bagasra O, Prockop DJ. Cultured adherent cells from marrow can serve as long-lasting precursor cells for bone, cartilage, and lung in irradiated mice. Proc Natl Acad Sci U S A 1995;92:4857-61.
201. Krause DS, Theise ND, Collector MI, Henegariu O, Hwang S, Gardner R, Neutzel S, Sharkis SJ. Multi-organ, multi-lineage engraftment by a single bone marrow-derived stem cell. Cell 2001;105:369-77.
202. Kotton DN, Ma BY, Cardoso WV, Sanderson EA, Summer RS, Williams MC, Fine A. Bone marrow-derived cells as progenitors of lung alveolar epithelium. Development 2001;128:5181-8.
203. Burnham E, Moss M. Progenitor cells in acute lung injury. Minerva Anestesiol 2006;72:369-74.
204. Lam CF, Liu YC, Hsu JK, Yeh PA, Su TY, Huang CC, Lin MW, Wu PC, Chang PJ, Tsai YC. Autologous transplantation of endothelial progenitor cells attenuates acute lung injury in rabbits. Anesthesiology 2008;108:392-401.

205. Weibel ER. Morphometric estimation of pulmonary diffusion capacity. I. Model and method. Respir Physiol 1970;11:54-75.
206. Kurahashi K, Kajikawa O, Sawa T, Ohara M, Gropper MA, Frank DW, Martin TR, Wiener-Kronish JP. Pathogenesis of septic shock in Pseudomonas aeruginosa pneumonia. J Clin Invest 1999;104:743-50.
207. Modelska K, Pittet JF, Folkesson HG, Courtney Broaddus V, Matthay MA. Acid-induced lung injury. Protective effect of anti-interleukin-8 pretreatment on alveolar epithelial barrier function in rabbits. Am J Respir Crit Care Med 1999;160:1450-6.
208. Sznajder JI. Strategies to increase alveolar epithelial fluid removal in the injured lung. Am J Respir Crit Care Med 1999;160:1441-2.
209. Mercer RR, Russell ML, Crapo JD. Alveolar septal structure in different species. J Appl Physiol 1994;77:1060-6.
210. Kollef MH, Schuster DP. The acute respiratory distress syndrome. N Engl J Med 1995;332:27-37.
211. Schuster DP, Kollef MH. Acute respiratory distress syndrome. Dis Mon 1996;42:270-326.
212. Lewis JF, Jobe AH. Surfactant and the adult respiratory distress syndrome. Am Rev Respir Dis 1993;147:218-33.
213. Wright JR. Immunoregulatory functions of surfactant proteins. Nat Rev Immunol 2005;5:58-68.
214. Greene KE, Wright JR, Steinberg KP, Ruzinski JT, Caldwell E, Wong WB, Hull W, Whitsett JA, Akino T, Kuroki Y, Nagae H, Hudson LD, Martin TR. Serial changes in surfactant-associated proteins in lung and serum before and after onset of ARDS. Am J Respir Crit Care Med 1999;160:1843-50.
215. Gregory TJ, Longmore WJ, Moxley MA, Whitsett JA, Reed CR, Fowler AA, 3rd, Hudson LD, Maunder RJ, Crim C, Hyers TM. Surfactant chemical composition and biophysical activity in acute respiratory distress syndrome. J Clin Invest 1991;88:1976-81.
216. Warr GA, Martin RR, Sharp PM, Rossen RD. Normal human bronchial immunoglobulins and proteins: effects of cigarette smoking. Am Rev Respir Dis 1977;116:25-30.
217. Low RB, Davis GS, Giancola MS. Biochemical analyses of bronchoalveolar lavage fluids of healthy human volunteer smokers and nonsmokers. Am Rev Respir Dis 1978;118:863-75.
218. Hunninghake GW, Gadek JE, Kawanami O, Ferrans VJ, Crystal RG. Inflammatory and immune processes in the human lung in health and disease: evaluation by bronchoalveolar lavage. Am J Pathol 1979;97:149-206.
219. Bell DY, Haseman JA, Spock A, McLennan G, Hook GE. Plasma proteins of the bronchoalveolar surface of the lungs of smokers and nonsmokers. Am Rev Respir Dis 1981;124:72-9.
220. Merrill WW, Naegel GP, Olchowski JJ, Reynolds HY. Immunoglobulin G subclass proteins in serum and lavage fluid of normal subjects. Quantitation and comparison with immunoglobulins A and E. Am Rev Respir Dis 1985;131:584-7.
221. Brigham KL, Staub NC. Pulmonary edema and acute lung injury research. Am J Respir Crit Care Med 1998;157:S109-13.

222. Fein A, Grossman RF, Jones JG, Overland E, Pitts L, Murray JF, Staub NC. The value of edema fluid protein measurement in patients with pulmonary edema. Am J Med 1979;67:32-8.
223. Taylor AE, Gaar KA, Jr. Estimation of equivalent pore radii of pulmonary capillary and alveolar membranes. Am J Physiol 1970;218:1133-40.
224. Berthiaume Y, Broaddus VC, Gropper MA, Tanita T, Matthay MA. Alveolar liquid and protein clearance from normal dog lungs. J Appl Physiol 1988;65:585-93.
225. Hastings RH, Grady M, Sakuma T, Matthay MA. Clearance of different-sized proteins from the alveolar space in humans and rabbits. J Appl Physiol 1992;73:1310-6.
226. Matthay MA, Folkesson HG, Clerici C. Lung epithelial fluid transport and the resolution of pulmonary edema. Physiol Rev 2002;82:569-600.
227. Matthay MA, Landolt CC, Staub NC. Differential liquid and protein clearance from the alveoli of anesthetized sheep. J Appl Physiol 1982;53:96-104.
228. Smedira N, Gates L, Hastings R, Jayr C, Sakuma T, Pittet JF, Matthay MA. Alveolar and lung liquid clearance in anesthetized rabbits. J Appl Physiol 1991;70:1827-35.
229. Folkesson HG, Matthay MA. Alveolar epithelial ion and fluid transport: recent progress. Am J Respir Cell Mol Biol 2006;35:10-9.
230. Vadasz I, Raviv S, Sznajder JI. Alveolar epithelium and Na,K-ATPase in acute lung injury. Intensive Care Med 2007;33:1243-51.
231. Sartori C, Matthay MA, Scherrer U. Transepithelial sodium and water transport in the lung. Major player and novel therapeutic target in pulmonary edema. Adv Exp Med Biol 2001;502:315-38.
232. Mutlu GM, Dumasius V, Burhop J, McShane PJ, Meng FJ, Welch L, Dumasius A, Mohebahmadi N, Thakuria G, Hardiman K, Matalon S, Hollenberg S, Factor P. Upregulation of alveolar epithelial active Na+ transport is dependent on beta2-adrenergic receptor signaling. Circ Res 2004;94:1091-100.
233. Factor P, Mutlu GM, Chen L, Mohameed J, Akhmedov AT, Meng FJ, Jilling T, Lewis ER, Johnson MD, Xu A, Kass D, Martino JM, Bellmeyer A, Albazi JS, Emala C, Lee HT, Dobbs LG, Matalon S. Adenosine regulation of alveolar fluid clearance. Proc Natl Acad Sci U S A 2007;104:4083-8.
234. Borok Z, Verkman AS. Lung edema clearance: 20 years of progress: invited review: role of aquaporin water channels in fluid transport in lung and airways. J Appl Physiol 2002;93:2199-206.
235. Verkman AS. Role of aquaporins in lung liquid physiology. Respir Physiol Neurobiol 2007;159:324-30.
236. Verghese GM, Ware LB, Matthay BA, Matthay MA. Alveolar epithelial fluid transport and the resolution of clinically severe hydrostatic pulmonary edema. J Appl Physiol 1999;87:1301-12.
237. Matthay MA, Wiener-Kronish JP. Intact epithelial barrier function is critical for the resolution of alveolar edema in humans. Am Rev Respir Dis 1990;142:1250-7.
238. Hagimoto N, Kuwano K, Miyazaki H, Kunitake R, Fujita M, Kawasaki M, Kaneko Y, Hara N. Induction of apoptosis and pulmonary fibrosis in mice in response to ligation of Fas antigen. Am J Respir Cell Mol Biol 1997;17:272-8.

239. Kuwano K. Involvement of epithelial cell apoptosis in interstitial lung diseases. Intern Med 2008;47:345-53.
240. Kuwano K, Kawasaki M, Maeyama T, Hagimoto N, Nakamura N, Shirakawa K, Hara N. Soluble form of fas and fas ligand in BAL fluid from patients with pulmonary fibrosis and bronchiolitis obliterans organizing pneumonia. Chest 2000;118:451-8.
241. Martin TR, Hagimoto N, Nakamura M, Matute-Bello G. Apoptosis and epithelial injury in the lungs. Proc Am Thorac Soc 2005;2:214-20.
242. Vlahakis NE, Schroeder MA, Limper AH, Hubmayr RD. Stretch induces cytokine release by alveolar epithelial cells in vitro. Am J Physiol 1999;277:L167-73.
243. Skerrett SJ, Liggitt HD, Hajjar AM, Ernst RK, Miller SI, Wilson CB. Respiratory epithelial cells regulate lung inflammation in response to inhaled endotoxin. Am J Physiol Lung Cell Mol Physiol 2004;287:L143-52.
244. Lum H, Malik AB. Regulation of vascular endothelial barrier function. Am J Physiol 1994;267:L223-41.
245. Ghitescu L, Fixman A, Simionescu M, Simionescu N. Specific binding sites for albumin restricted to plasmalemmal vesicles of continuous capillary endothelium: receptor-mediated transcytosis. J Cell Biol 1986;102:1304-11.
246. Fishman JB, Rubin JB, Handrahan JV, Connor JR, Fine RE. Receptor-mediated transcytosis of transferrin across the blood-brain barrier. J Neurosci Res 1987;18:299-304.
247. Kataoka M, Tavassoli M. The role of liver endothelium in the binding and uptake of ceruloplasmin: studies with colloidal gold probe. J Ultrastruct Res 1985;90:194-202.
248. Mommaas-Kienhuis AM, Krijbolder LH, Van Hinsbergh VW, Daems WT, Vermeer BJ. Visualization of binding and receptor-mediated uptake of low density lipoproteins by human endothelial cells. Eur J Cell Biol 1985;36:201-8.
249. Mineo C, Yagyu Y, Imanaka T, Takano T. Transcellular transport of angiotensin II through a cultured arterial endothelial monolayer. Exp Cell Res 1990;190:99-103.
250. Predescu D, Predescu S, McQuistan T, Palade GE. Transcytosis of alpha1-acidic glycoprotein in the continuous microvascular endothelium. Proc Natl Acad Sci U S A 1998;95:6175-80.
251. Kuebler WM, Parthasarathi K, Wang PM, Bhattacharya J. A novel signaling mechanism between gas and blood compartments of the lung. J Clin Invest 2000;106:607.
252. Kuebler WM, Ying X, Singh B, Issekutz AC, Bhattacharya J. Pressure is proinflammatory in lung venular capillaries. J Clin Invest 1999;104:495-502.
253. Dudek SM, Garcia JG. Cytoskeletal regulation of pulmonary vascular permeability. J Appl Physiol 2001;91:1487-500.
254. Garcia JG, Schaphorst KL. Regulation of endothelial cell gap formation and paracellular permeability. J Investig Med 1995;43:117-26.
255. Dudek SM, Jacobson JR, Chiang ET, Birukov KG, Wang P, Zhan X, Garcia JG. Pulmonary endothelial cell barrier enhancement by sphingosine 1-phosphate: roles for cortactin and myosin light chain kinase. J Biol Chem 2004;279:24692-700.

256. Finigan JH, Dudek SM, Singleton PA, Chiang ET, Jacobson JR, Camp SM, Ye SQ, Garcia JG. Activated protein C mediates novel lung endothelial barrier enhancement: role of sphingosine 1-phosphate receptor transactivation. J Biol Chem 2005;280:17286-93.
257. Zimmerman GA, McIntyre TM, Prescott SM. Adhesion and signaling in vascular cell--cell interactions. J Clin Invest 1996;98:1699-702.
258. Martin TR, Nakamura M, Matute-Bello G. The role of apoptosis in acute lung injury. Crit Care Med 2003;31:S184-8.
259. Tang PS, Mura M, Seth R, Liu M. Acute lung injury and cell death: how many ways can cells die? Am J Physiol Lung Cell Mol Physiol 2008;294:L632-41.
260. Han SI, Kim YS, Kim TH. Role of apoptotic and necrotic cell death under physiologic conditions. BMB Rep 2008;41:1-10.
261. Lockshin RA, Zakeri Z. Cell death in health and disease. J Cell Mol Med 2007;11:1214-24.
262. Wang X, Ryter SW, Dai C, Tang ZL, Watkins SC, Yin XM, Song R, Choi AM. Necrotic cell death in response to oxidant stress involves the activation of the apoptogenic caspase-8/bid pathway. J Biol Chem 2003;278:29184-91.
263. Seeger W, Birkemeyer RG, Ermert L, Suttorp N, Bhakdi S, Duncker HR. Staphylococcal alpha-toxin-induced vascular leakage in isolated perfused rabbit lungs. Lab Invest 1990;63:341-9.
264. Sato H, Frank DW, Hillard CJ, Feix JB, Pankhaniya RR, Moriyama K, Finck-Barbancon V, Buchaklian A, Lei M, Long RM, Wiener-Kronish J, Sawa T. The mechanism of action of the Pseudomonas aeruginosa-encoded type III cytotoxin, ExoU. EMBO J 2003;22:2959-69.
265. Plotz FB, Slutsky AS, van Vught AJ, Heijnen CJ. Ventilator-induced lung injury and multiple system organ failure: a critical review of facts and hypotheses. Intensive Care Med 2004;30:1865-72.
266. Kerr JF, Wyllie AH, Currie AR. Apoptosis: a basic biological phenomenon with wide-ranging implications in tissue kinetics. Br J Cancer 1972;26:239-57.
267. Wyllie AH, Kerr JF, Currie AR. Cell death: the significance of apoptosis. Int Rev Cytol 1980;68:251-306.
268. Wang R, Zagariya A, Ang E, Ibarra-Sunga O, Uhal BD. Fas-induced apoptosis of alveolar epithelial cells requires ANG II generation and receptor interaction. Am J Physiol 1999;277:L1245-50.
269. Hotchkiss RS, Nicholson DW. Apoptosis and caspases regulate death and inflammation in sepsis. Nat Rev Immunol 2006;6:813-22.
270. Tinsley KW, Grayson MH, Swanson PE, Drewry AM, Chang KC, Karl IE, Hotchkiss RS. Sepsis induces apoptosis and profound depletion of splenic interdigitating and follicular dendritic cells. J Immunol 2003;171:909-14.
271. Oberholzer C, Oberholzer A, Clare-Salzler M, Moldawer LL. Apoptosis in sepsis: a new target for therapeutic exploration. FASEB J 2001;15:879-92.
272. Bardales RH, Xie SS, Schaefer RF, Hsu SM. Apoptosis is a major pathway responsible for the resolution of type II pneumocytes in acute lung injury. Am J Pathol 1996;149:845-52.
273. Guinee D, Jr., Brambilla E, Fleming M, Hayashi T, Rahn M, Koss M, Ferrans V, Travis W. The potential role of BAX and BCL-2 expression in diffuse alveolar damage. Am J Pathol 1997;151:999-1007.

274. Kitamura Y, Hashimoto S, Mizuta N, Kobayashi A, Kooguchi K, Fujiwara I, Nakajima H. Fas/FasL-dependent apoptosis of alveolar cells after lipopolysaccharide-induced lung injury in mice. Am J Respir Crit Care Med 2001;163:762-9.
275. Vernooy JH, Dentener MA, van Suylen RJ, Buurman WA, Wouters EF. Intratracheal instillation of lipopolysaccharide in mice induces apoptosis in bronchial epithelial cells: no role for tumor necrosis factor-alpha and infiltrating neutrophils. Am J Respir Cell Mol Biol 2001;24:569-76.
276. Kawasaki M, Kuwano K, Hagimoto N, Matsuba T, Kunitake R, Tanaka T, Maeyama T, Hara N. Protection from lethal apoptosis in lipopolysaccharide-induced acute lung injury in mice by a caspase inhibitor. Am J Pathol 2000;157:597-603.
277. Fujita M, Kuwano K, Kunitake R, Hagimoto N, Miyazaki H, Kaneko Y, Kawasaki M, Maeyama T, Hara N. Endothelial cell apoptosis in lipopolysaccharide-induced lung injury in mice. Int Arch Allergy Immunol 1998;117:202-8.
278. Martin TR. Neutrophils and lung injury: getting it right. J Clin Invest 2002;110:1603-5.
279. Abraham E. Neutrophils and acute lung injury. Crit Care Med 2003;31:S195-9.
280. Wen LP, Madani K, Fahrni JA, Duncan SR, Rosen GD. Dexamethasone inhibits lung epithelial cell apoptosis induced by IFN-gamma and Fas. Am J Physiol 1997;273:L921-9.
281. Nagata S, Golstein P. The Fas death factor. Science 1995;267:1449-56.
282. Matute-Bello G, Winn RK, Jonas M, Chi EY, Martin TR, Liles WC. Fas (CD95) induces alveolar epithelial cell apoptosis in vivo: implications for acute pulmonary inflammation. Am J Pathol 2001;158:153-61.
283. Fine A, Anderson NL, Rothstein TL, Williams MC, Gochuico BR. Fas expression in pulmonary alveolar type II cells. Am J Physiol 1997;273:L64-71.
284. Matute-Bello G, Liles WC, Steinberg KP, Kiener PA, Mongovin S, Chi EY, Jonas M, Martin TR. Soluble Fas ligand induces epithelial cell apoptosis in humans with acute lung injury (ARDS). J Immunol 1999;163:2217-25.
285. Wang R, Zagariya A, Ibarra-Sunga O, Gidea C, Ang E, Deshmukh S, Chaudhary G, Baraboutis J, Filippatos G, Uhal BD. Angiotensin II induces apoptosis in human and rat alveolar epithelial cells. Am J Physiol 1999;276:L885-9.
286. Idell S, Kueppers F, Lippmann M, Rosen H, Niederman M, Fein A. Angiotensin converting enzyme in bronchoalveolar lavage in ARDS. Chest 1987;91:52-6.
287. Vazquez de Lara L, Becerril C, Montano M, Ramos C, Maldonado V, Melendez J, Phelps DS, Pardo A, Selman M. Surfactant components modulate fibroblast apoptosis and type I collagen and collagenase-1 expression. Am J Physiol Lung Cell Mol Physiol 2000;279:L950-7.
288. White MK, Baireddy V, Strayer DS. Natural protection from apoptosis by surfactant protein A in type II pneumocytes. Exp Cell Res 2001;263:183-92.
289. Matute-Bello G, Martin TR. Science review: apoptosis in acute lung injury. Crit Care 2003;7:355-8.
290. Shi Y. A structural view of mitochondria-mediated apoptosis. Nat Struct Biol 2001;8:394-401.

291. Buccellato LJ, Tso M, Akinci OI, Chandel NS, Budinger GR. Reactive oxygen species are required for hyperoxia-induced Bax activation and cell death in alveolar epithelial cells. J Biol Chem 2004;279:6753-60.
292. Krick S, Eul BG, Hanze J, Savai R, Grimminger F, Seeger W, Rose F. Role of hypoxia-inducible factor-1alpha in hypoxia-induced apoptosis of primary alveolar epithelial type II cells. Am J Respir Cell Mol Biol 2005;32:395-403.
293. Hagimoto N, Kuwano K, Nomoto Y, Kunitake R, Hara N. Apoptosis and expression of Fas/Fas ligand mRNA in bleomycin-induced pulmonary fibrosis in mice. Am J Respir Cell Mol Biol 1997;16:91-101.
294. Uhal BD. Epithelial apoptosis in the initiation of lung fibrosis. Eur Respir J Suppl 2003;44:7s-9s.
295. Kuwano K, Hagimoto N, Kawasaki M, Yatomi T, Nakamura N, Nagata S, Suda T, Kunitake R, Maeyama T, Miyazaki H, Hara N. Essential roles of the Fas-Fas ligand pathway in the development of pulmonary fibrosis. J Clin Invest 1999;104:13-9.
296. Matute-Bello G, Wurfel MM, Lee JS, Park DR, Frevert CW, Madtes DK, Shapiro SD, Martin TR. Essential role of MMP-12 in Fas-induced lung fibrosis. Am J Respir Cell Mol Biol 2007;37:210-21.
297. Casciola-Rosen LA, Anhalt G, Rosen A. Autoantigens targeted in systemic lupus erythematosus are clustered in two populations of surface structures on apoptotic keratinocytes. J Exp Med 1994;179:1317-30.
298. Chang MK, Binder CJ, Miller YI, Subbanagounder G, Silverman GJ, Berliner JA, Witztum JL. Apoptotic cells with oxidation-specific epitopes are immunogenic and proinflammatory. J Exp Med 2004;200:1359-70.
299. Grigg JM, Savill JS, Sarraf C, Haslett C, Silverman M. Neutrophil apoptosis and clearance from neonatal lungs. Lancet 1991;338:720-2.
300. Haslett C, Savill JS, Whyte MK, Stern M, Dransfield I, Meagher LC. Granulocyte apoptosis and the control of inflammation. Philos Trans R Soc Lond B Biol Sci 1994;345:327-33.
301. Hussain N, Wu F, Zhu L, Thrall RS, Kresch MJ. Neutrophil apoptosis during the development and resolution of oleic acid-induced acute lung injury in the rat. Am J Respir Cell Mol Biol 1998;19:867-74.
302. Morimoto K, Amano H, Sonoda F, Baba M, Senba M, Yoshimine H, Yamamoto H, Ii T, Oishi K, Nagatake T. Alveolar macrophages that phagocytose apoptotic neutrophils produce hepatocyte growth factor during bacterial pneumonia in mice. Am J Respir Cell Mol Biol 2001;24:608-15.
303. Parsey MV, Kaneko D, Shenkar R, Abraham E. Neutrophil apoptosis in the lung after hemorrhage or endotoxemia: apoptosis and migration are independent of IL-1beta. Clin Immunol 1999;91:219-25.
304. Savill J, Haslett C. Granulocyte clearance by apoptosis in the resolution of inflammation. Semin Cell Biol 1995;6:385-93.
305. Watson RW, Rotstein OD, Parodo J, Jimenez M, Soric I, Bitar R, Marshall JC. Impaired apoptotic death signaling in inflammatory lung neutrophils is associated with decreased expression of interleukin-1 beta converting enzyme family proteases (caspases). Surgery 1997;122:163-71; discussion 171-2.
306. Rowe SJ, Allen L, Ridger VC, Hellewell PG, Whyte MK. Caspase-1-deficient mice have delayed neutrophil apoptosis and a prolonged inflammatory response to lipopolysaccharide-induced acute lung injury. J Immunol 2002;169:6401-7.

307. El Kebir D, Jozsef L, Pan W, Filep JG. Myeloperoxidase delays neutrophil apoptosis through CD11b/CD18 integrins and prolongs inflammation. Circ Res 2008;103:352-9.
308. Hu M, Lin X, Du Q, Miller EJ, Wang P, Simms HH. Regulation of polymorphonuclear leukocyte apoptosis: role of lung endothelium-epithelium bilayer transmigration. Am J Physiol Lung Cell Mol Physiol 2005;288:L266-74.
309. Kupfner JG, Arcaroli JJ, Yum HK, Nadler SG, Yang KY, Abraham E. Role of NF-kappaB in endotoxemia-induced alterations of lung neutrophil apoptosis. J Immunol 2001;167:7044-51.
310. Liu Y, Zhang J, Zhao Z, Ling Y. Pro-apoptotic role of NF-kappaB pathway inhibition in lipopolysaccharide-stimulated polymorphonuclear neutrophils. Chin Med J (Engl) 2003;116:1257-61.
311. Aggarwal A, Baker CS, Evans TW, Haslam PL. G-CSF and IL-8 but not GM-CSF correlate with severity of pulmonary neutrophilia in acute respiratory distress syndrome. Eur Respir J 2000;15:895-901.
312. Lesur O, Kokis A, Hermans C, Fulop T, Bernard A, Lane D. Interleukin-2 involvement in early acute respiratory distress syndrome: relationship with polymorphonuclear neutrophil apoptosis and patient survival. Crit Care Med 2000;28:3814-22.
313. Goodman ER, Kleinstein E, Fusco AM, Quinlan DP, Lavery R, Livingston DH, Deitch EA, Hauser CJ. Role of interleukin 8 in the genesis of acute respiratory distress syndrome through an effect on neutrophil apoptosis. Arch Surg 1998;133:1234-9.
314. Lu Q, Harrington EO, Rounds S. Apoptosis and lung injury. Keio J Med 2005;54:184-9.
315. Kotecha S, Mildner RJ, Prince LR, Vyas JR, Currie AE, Lawson RA, Whyte MK. The role of neutrophil apoptosis in the resolution of acute lung injury in newborn infants. Thorax 2003;58:961-7.
316. Liu R, Xiao N, Tian K. [The role of PMN apoptosis delay in acute lung injury induced by administrating LPS]. Zhonghua Yi Xue Za Zhi 2001;81:617-21.
317. Pugin J, Verghese G, Widmer MC, Matthay MA. The alveolar space is the site of intense inflammatory and profibrotic reactions in the early phase of acute respiratory distress syndrome. Crit Care Med 1999;27:304-12.
318. Lesur O, Berthiaume Y, Blaise G, Damas P, Deland E, Guimond JG, Michel RP. Acute respiratory distress syndrome: 30 years later. Can Respir J 1999;6:71-86.
319. Martin TR. Lung cytokines and ARDS: Roger S. Mitchell Lecture. Chest 1999;116:2S-8S.
320. O'Grady NP, Preas HL, Pugin J, Fiuza C, Tropea M, Reda D, Banks SM, Suffredini AF. Local inflammatory responses following bronchial endotoxin instillation in humans. Am J Respir Crit Care Med 2001;163:1591-8.
321. Goodman RB, Pugin J, Lee JS, Matthay MA. Cytokine-mediated inflammation in acute lung injury. Cytokine Growth Factor Rev 2003;14:523-35.
322. Armstrong L, Millar AB. Relative production of tumour necrosis factor alpha and interleukin 10 in adult respiratory distress syndrome. Thorax 1997;52:442-6.
323. Geiser T, Jarreau PH, Atabai K, Matthay MA. Interleukin-1beta augments in vitro alveolar epithelial repair. Am J Physiol Lung Cell Mol Physiol 2000;279:L1184-90.

324. Prabhakaran P, Ware LB, White KE, Cross MT, Matthay MA, Olman MA. Elevated levels of plasminogen activator inhibitor-1 in pulmonary edema fluid are associated with mortality in acute lung injury. Am J Physiol Lung Cell Mol Physiol 2003;285:L20-8.
325. Chollet-Martin S. Polymorphonuclear neutrophil activation during the acute respiratory distress syndrome. Intensive Care Med 2000;26:1575-7.
326. Chollet-Martin S, Jourdain B, Gibert C, Elbim C, Chastre J, Gougerot-Pocidalo MA. Interactions between neutrophils and cytokines in blood and alveolar spaces during ARDS. Am J Respir Crit Care Med 1996;154:594-601.
327. Martin TR. Cytokines and lung injury: searching for useful biomarkers. Crit Care Med 2005;33:230-2.
328. McClintock D, Zhuo H, Wickersham N, Matthay MA, Ware LB. Biomarkers of inflammation, coagulation and fibrinolysis predict mortality in acute lung injury. Crit Care 2008;12:R41.
329. Aldridge AJ. Role of the neutrophil in septic shock and the adult respiratory distress syndrome. Eur J Surg 2002;168:204-14.
330. Lee WL, Downey GP. Neutrophil activation and acute lung injury. Curr Opin Crit Care 2001;7:1-7.
331. Coldren CD, Nick JA, Poch KR, Woolum MD, Fouty BW, O'Brien JM, Gruber MP, Zamora MR, Svetkauskaite D, Richter DA, He Q, Park JS, Overdier KH, Abraham E, Geraci MW. Functional and genomic changes induced by alveolar transmigration in human neutrophils. Am J Physiol Lung Cell Mol Physiol 2006;291:L1267-76.
332. Chow CW, Herrera Abreu MT, Suzuki T, Downey GP. Oxidative stress and acute lung injury. Am J Respir Cell Mol Biol 2003;29:427-31.
333. Heflin AC, Jr., Brigham KL. Prevention by granulocyte depletion of increased vascular permeability of sheep lung following endotoxemia. J Clin Invest 1981;68:1253-60.
334. Winn R, Maunder R, Chi E, Harlan J. Neutrophil depletion does not prevent lung edema after endotoxin infusion in goats. J Appl Physiol 1987;62:116-21.
335. Laufe MD, Simon RH, Flint A, Keller JB. Adult respiratory distress syndrome in neutropenic patients. Am J Med 1986;80:1022-6.
336. Martin TR, Pistorese BP, Chi EY, Goodman RB, Matthay MA. Effects of leukotriene B4 in the human lung. Recruitment of neutrophils into the alveolar spaces without a change in protein permeability. J Clin Invest 1989;84:1609-19.
337. Braude S, Apperley J, Krausz T, Goldman JM, Royston D. Adult respiratory distress syndrome after allogeneic bone-marrow transplantation: evidence for a neutrophil-independent mechanism. Lancet 1985;1:1239-42.
338. Weiland JE, Davis WB, Holter JF, Mohammed JR, Dorinsky PM, Gadek JE. Lung neutrophils in the adult respiratory distress syndrome. Clinical and pathophysiologic significance. Am Rev Respir Dis 1986;133:218-25.
339. Chua F, Dunsmore SE, Clingen PH, Mutsaers SE, Shapiro SD, Segal AW, Roes J, Laurent GJ. Mice lacking neutrophil elastase are resistant to bleomycin-induced pulmonary fibrosis. Am J Pathol 2007;170:65-74.
340. Bellingan GJ. The pulmonary physician in critical care * 6: The pathogenesis of ALI/ARDS. Thorax 2002;57:540-6.

341. Pugin J, Ricou B, Steinberg KP, Suter PM, Martin TR. Proinflammatory activity in bronchoalveolar lavage fluids from patients with ARDS, a prominent role for interleukin-1. Am J Respir Crit Care Med 1996;153:1850-6.
342. Donnelly SC, Strieter RM, Kunkel SL, Walz A, Robertson CR, Carter DC, Grant IS, Pollok AJ, Haslett C. Interleukin-8 and development of adult respiratory distress syndrome in at-risk patient groups. Lancet 1993;341:643-7.
343. Hyers TM, Tricomi SM, Dettenmeier PA, Fowler AA. Tumor necrosis factor levels in serum and bronchoalveolar lavage fluid of patients with the adult respiratory distress syndrome. Am Rev Respir Dis 1991;144:268-71.
344. Jacobs RF, Tabor DR, Burks AW, Campbell GD. Elevated interleukin-1 release by human alveolar macrophages during the adult respiratory distress syndrome. Am Rev Respir Dis 1989;140:1686-92.
345. Meduri GU, Kohler G, Headley S, Tolley E, Stentz F, Postlethwaite A. Inflammatory cytokines in the BAL of patients with ARDS. Persistent elevation over time predicts poor outcome. Chest 1995;108:1303-14.
346. Goodman RB, Strieter RM, Martin DP, Steinberg KP, Milberg JA, Maunder RJ, Kunkel SL, Walz A, Hudson LD, Martin TR. Inflammatory cytokines in patients with persistence of the acute respiratory distress syndrome. Am J Respir Crit Care Med 1996;154:602-11.
347. Geiser T, Atabai K, Jarreau PH, Ware LB, Pugin J, Matthay MA. Pulmonary edema fluid from patients with acute lung injury augments in vitro alveolar epithelial repair by an IL-1beta-dependent mechanism. Am J Respir Crit Care Med 2001;163:1384-8.
348. Kolb M, Margetts PJ, Anthony DC, Pitossi F, Gauldie J. Transient expression of IL-1beta induces acute lung injury and chronic repair leading to pulmonary fibrosis. J Clin Invest 2001;107:1529-36.
349. Bonniaud P, Margetts PJ, Ask K, Flanders K, Gauldie J, Kolb M. TGF-beta and Smad3 signaling link inflammation to chronic fibrogenesis. J Immunol 2005;175:5390-5.
350. Leff JA, Baer JW, Bodman ME, Kirkman JM, Shanley PF, Patton LM, Beehler CJ, McCord JM, Repine JE. Interleukin-1-induced lung neutrophil accumulation and oxygen metabolite-mediated lung leak in rats. Am J Physiol 1994;266:L2-8.
351. Frank J, Roux J, Kawakatsu H, Su G, Dagenais A, Berthiaume Y, Howard M, Canessa CM, Fang X, Sheppard D, Matthay MA, Pittet JF. Transforming growth factor-beta1 decreases expression of the epithelial sodium channel alphaENaC and alveolar epithelial vectorial sodium and fluid transport via an ERK1/2-dependent mechanism. J Biol Chem 2003;278:43939-50.
352. Villard J, Dayer-Pastore F, Hamacher J, Aubert JD, Schlegel-Haueter S, Nicod LP. GRO alpha and interleukin-8 in Pneumocystis carinii or bacterial pneumonia and adult respiratory distress syndrome. Am J Respir Crit Care Med 1995;152:1549-54.
353. Parsons PE, Fowler AA, Hyers TM, Henson PM. Chemotactic activity in bronchoalveolar lavage fluid from patients with adult respiratory distress syndrome. Am Rev Respir Dis 1985;132:490-3.
354. Donnelly SC, Strieter RM, Kunkel SL, Walz A, Steedman D, Grant IS, Pollok AJ, Carter DC, Haslett C. Chemotactic cytokines in the established adult respiratory distress syndrome and at-risk patients. Chest 1994;105:98S-99S.

355. Donnelly SC, Strieter RM, Reid PT, Kunkel SL, Burdick MD, Armstrong I, Mackenzie A, Haslett C. The association between mortality rates and decreased concentrations of interleukin-10 and interleukin-1 receptor antagonist in the lung fluids of patients with the adult respiratory distress syndrome. Ann Intern Med 1996;125:191-6.
356. Beutler B. Toll-like receptors: how they work and what they do. Curr Opin Hematol 2002;9:2-10.
357. Martin TR. Recognition of bacterial endotoxin in the lungs. Am J Respir Cell Mol Biol 2000;23:128-32.
358. Martin TR, Rubenfeld GD, Ruzinski JT, Goodman RB, Steinberg KP, Leturcq DJ, Moriarty AM, Raghu G, Baughman RP, Hudson LD. Relationship between soluble CD14, lipopolysaccharide binding protein, and the alveolar inflammatory response in patients with acute respiratory distress syndrome. Am J Respir Crit Care Med 1997;155:937-44.
359. Martin TR, Mathison JC, Tobias PS, Leturcq DJ, Moriarty AM, Maunder RJ, Ulevitch RJ. Lipopolysaccharide binding protein enhances the responsiveness of alveolar macrophages to bacterial lipopolysaccharide. Implications for cytokine production in normal and injured lungs. J Clin Invest 1992;90:2209-19.
360. Nathan CF. Secretory products of macrophages. J Clin Invest 1987;79:319-26.
361. Demedts IK, Brusselle GG, Bracke KR, Vermaelen KY, Pauwels RA. Matrix metalloproteinases in asthma and COPD. Curr Opin Pharmacol 2005;5:257-63.
362. Parks WC. Matrix metalloproteinases in lung repair. Eur Respir J Suppl 2003;44:36s-38s.
363. Parks WC, Shapiro SD. Matrix metalloproteinases in lung biology. Respir Res 2001;2:10-9.
364. Parks WC, Wilson CL, Lopez-Boado YS. Matrix metalloproteinases as modulators of inflammation and innate immunity. Nat Rev Immunol 2004;4:617-29.
365. Shapiro SD, Senior RM. Matrix metalloproteinases. Matrix degradation and more. Am J Respir Cell Mol Biol 1999;20:1100-2.
366. Repine JE, Beehler CJ. Neutrophils and adult respiratory distress syndrome: two interlocking perspectives in 1991. Am Rev Respir Dis 1991;144:251-2.
367. Atkinson JJ, Holmbeck K, Yamada S, Birkedal-Hansen H, Parks WC, Senior RM. Membrane-type 1 matrix metalloproteinase is required for normal alveolar development. Dev Dyn 2005;232:1079-90.
368. Atkinson JJ, Senior RM. Matrix metalloproteinase-9 in lung remodeling. Am J Respir Cell Mol Biol 2003;28:12-24.
369. Lagente V, Manoury B, Nenan S, Le Quement C, Martin-Chouly C, Boichot E. Role of matrix metalloproteinases in the development of airway inflammation and remodeling. Braz J Med Biol Res 2005;38:1521-30.
370. Fligiel SE, Standiford T, Fligiel HM, Tashkin D, Strieter RM, Warner RL, Johnson KJ, Varani J. Matrix metalloproteinases and matrix metalloproteinase inhibitors in acute lung injury. Hum Pathol 2006;37:422-30.
371. Gomez DE, Alonso DF, Yoshiji H, Thorgeirsson UP. Tissue inhibitors of metalloproteinases: structure, regulation and biological functions. Eur J Cell Biol 1997;74:111-22.
372. Vignola AM, Riccobono L, Mirabella A, Profita M, Chanez P, Bellia V, Mautino G, D'Accardi P, Bousquet J, Bonsignore G. Sputum metalloproteinase-

9/tissue inhibitor of metalloproteinase-1 ratio correlates with airflow obstruction in asthma and chronic bronchitis. Am J Respir Crit Care Med 1998;158:1945-50.

373. Hayashi T, Stetler-Stevenson WG, Fleming MV, Fishback N, Koss MN, Liotta LA, Ferrans VJ, Travis WD. Immunohistochemical study of metalloproteinases and their tissue inhibitors in the lungs of patients with diffuse alveolar damage and idiopathic pulmonary fibrosis. Am J Pathol 1996;149:1241-56.
374. Ohno I, Ohtani H, Nitta Y, Suzuki J, Hoshi H, Honma M, Isoyama S, Tanno Y, Tamura G, Yamauchi K, Nagura H, Shirato K. Eosinophils as a source of matrix metalloproteinase-9 in asthmatic airway inflammation. Am J Respir Cell Mol Biol 1997;16:212-9.
375. Johnson D, Thomson D, Hurst T, Prasad K, Wilson T, Murphy F, Saxena A, Mayers I. Neutrophil-mediated acute lung injury after extracorporeal perfusion. J Thorac Cardiovasc Surg 1994;107:1193-202.
376. Tonz M, Mihaljevic T, von Segesser LK, Fehr J, Schmid ER, Turina MI. Acute lung injury during cardiopulmonary bypass. Are the neutrophils responsible? Chest 1995;108:1551-6.
377. Sweet DG, McMahon KJ, Curley AE, O'Connor CM, Halliday HL. Type I collagenases in bronchoalveolar lavage fluid from preterm babies at risk of developing chronic lung disease. Arch Dis Child Fetal Neonatal Ed 2001;84:F168-71.
378. Cederqvist K, Sorsa T, Tervahartiala T, Maisi P, Reunanen K, Lassus P, Andersson S. Matrix metalloproteinases-2, -8, and -9 and TIMP-2 in tracheal aspirates from preterm infants with respiratory distress. Pediatrics 2001;108:686-92.
379. Hartog CM, Wermelt JA, Sommerfeld CO, Eichler W, Dalhoff K, Braun J. Pulmonary matrix metalloproteinase excess in hospital-acquired pneumonia. Am J Respir Crit Care Med 2003;167:593-8.
380. Gibbs DF, Shanley TP, Warner RL, Murphy HS, Varani J, Johnson KJ. Role of matrix metalloproteinases in models of macrophage-dependent acute lung injury. Evidence for alveolar macrophage as source of proteinases. Am J Respir Cell Mol Biol 1999;20:1145-54.
381. Gibbs DF, Warner RL, Weiss SJ, Johnson KJ, Varani J. Characterization of matrix metalloproteinases produced by rat alveolar macrophages. Am J Respir Cell Mol Biol 1999;20:1136-44.
382. Mulligan MS, Desrochers PE, Chinnaiyan AM, Gibbs DF, Varani J, Johnson KJ, Weiss SJ. In vivo suppression of immune complex-induced alveolitis by secretory leukoproteinase inhibitor and tissue inhibitor of metalloproteinases 2. Proc Natl Acad Sci U S A 1993;90:11523-7.
383. Varani J, Ward PA. Mechanisms of endothelial cell injury in acute inflammation. Shock 1994;2:311-9.
384. Li Q, Park PW, Wilson CL, Parks WC. Matrilysin shedding of syndecan-1 regulates chemokine mobilization and transepithelial efflux of neutrophils in acute lung injury. Cell 2002;111:635-46.
385. Warner RL, Beltran L, Younkin EM, Lewis CS, Weiss SJ, Varani J, Johnson KJ. Role of stromelysin 1 and gelatinase B in experimental acute lung injury. Am J Respir Cell Mol Biol 2001;24:537-44.

386. Baragi VM, Fliszar CJ, Conroy MC, Ye QZ, Shipley JM, Welgus HG. Contribution of the C-terminal domain of metalloproteinases to binding by tissue inhibitor of metalloproteinases. C-terminal truncated stromelysin and matrilysin exhibit equally compromised binding affinities as compared to full-length stromelysin. J Biol Chem 1994;269:12692-7.
387. Wilson CL, Matrisian LM. Matrilysin: an epithelial matrix metalloproteinase with potentially novel functions. Int J Biochem Cell Biol 1996;28:123-36.
388. Gearing AJ, Beckett P, Christodoulou M, Churchill M, Clements J, Davidson AH, Drummond AH, Galloway WA, Gilbert R, Gordon JL, et al. Processing of tumour necrosis factor-alpha precursor by metalloproteinases. Nature 1994;370:555-7.
389. Wilson CL, Heppner KJ, Rudolph LA, Matrisian LM. The metalloproteinase matrilysin is preferentially expressed by epithelial cells in a tissue-restricted pattern in the mouse. Mol Biol Cell 1995;6:851-69.
390. Saarialho-Kere UK, Crouch EC, Parks WC. Matrix metalloproteinase matrilysin is constitutively expressed in adult human exocrine epithelium. J Invest Dermatol 1995;105:190-6.
391. Dunsmore SE, Saarialho-Kere UK, Roby JD, Wilson CL, Matrisian LM, Welgus HG, Parks WC. Matrilysin expression and function in airway epithelium. J Clin Invest 1998;102:1321-31.
392. Lopez-Boado YS, Wilson CL, Parks WC. Regulation of matrilysin expression in airway epithelial cells by Pseudomonas aeruginosa flagellin. J Biol Chem 2001;276:41417-23.
393. Zuo F, Kaminski N, Eugui E, Allard J, Yakhini Z, Ben-Dor A, Lollini L, Morris D, Kim Y, DeLustro B, Sheppard D, Pardo A, Selman M, Heller RA. Gene expression analysis reveals matrilysin as a key regulator of pulmonary fibrosis in mice and humans. Proc Natl Acad Sci U S A 2002;99:6292-7.
394. Cosgrove GP, Schwarz MI, Geraci MW, Brown KK, Worthen GS. Overexpression of matrix metalloproteinase-7 in pulmonary fibrosis. Chest 2002;121:25S-26S.
395. McGuire JK, Li Q, Parks WC. Matrilysin (matrix metalloproteinase-7) mediates E-cadherin ectodomain shedding in injured lung epithelium. Am J Pathol 2003;162:1831-43.
396. Idell S, Koenig KB, Fair DS, Martin TR, McLarty J, Maunder RJ. Serial abnormalities of fibrin turnover in evolving adult respiratory distress syndrome. Am J Physiol 1991;261:L240-8.
397. Idell S. Extravascular coagulation and fibrin deposition in acute lung injury. New Horiz 1994;2:566-74.
398. Nagy Z, Kolev K, Csonka E, Pek M, Machovich R. Contraction of human brain endothelial cells induced by thrombogenic and fibrinolytic factors. An in vitro cell culture model. Stroke 1995;26:265-70.
399. Grinnell F, Feld M, Minter D. Fibroblast adhesion to fibrinogen and fibrin substrata: requirement for cold-insoluble globulin (plasma fibronectin). Cell 1980;19:517-25.
400. Fukuda Y, Ishizaki M, Masuda Y, Kimura G, Kawanami O, Masugi Y. The role of intraalveolar fibrosis in the process of pulmonary structural remodeling in patients with diffuse alveolar damage. Am J Pathol 1987;126:171-82.

401. Hesselvik JF, Malm J, Dahlback B, Blomback M. Protein C, protein S and C4b-binding protein in severe infection and septic shock. Thromb Haemost 1991;65:126-9.
402. Yan SB, Helterbrand JD, Hartman DL, Wright TJ, Bernard GR. Low levels of protein C are associated with poor outcome in severe sepsis. Chest 2001;120:915-22.
403. Derhaschnig U, Reiter R, Knobl P, Baumgartner M, Keen P, Jilma B. Recombinant human activated protein C (rhAPC; drotrecogin alfa [activated]) has minimal effect on markers of coagulation, fibrinolysis, and inflammation in acute human endotoxemia. Blood 2003;102:2093-8.
404. Kalil AC, Coyle SM, Um JY, LaRosa SP, Turlo MA, Calvano SE, Sundin DP, Nelson DR, Lowry SF. Effects of drotrecogin alfa (activated) in human endotoxemia. Shock 2004;21:222-9.
405. Fink MP. Role of reactive oxygen and nitrogen species in acute respiratory distress syndrome. Curr Opin Crit Care 2002;8:6-11.
406. Ischiropoulos H. Biological tyrosine nitration: a pathophysiological function of nitric oxide and reactive oxygen species. Arch Biochem Biophys 1998;356:1-11.
407. Schraufstatter IU, Hyslop PA, Jackson J, Revak SD, Cochrane CC. Oxidant and protease injury of the lung. Bull Eur Physiopathol Respir 1987;23:297-302.
408. Schraufstatter IU, Revak SD, Cochrane CG. Proteases and oxidants in experimental pulmonary inflammatory injury. J Clin Invest 1984;73:1175-84.
409. Bunnell E, Pacht ER. Oxidized glutathione is increased in the alveolar fluid of patients with the adult respiratory distress syndrome. Am Rev Respir Dis 1993;148:1174-8.
410. Rahman I, MacNee W. Regulation of redox glutathione levels and gene transcription in lung inflammation: therapeutic approaches. Free Radic Biol Med 2000;28:1405-20.
411. Terada LS, Dormish JJ, Shanley PF, Leff JA, Anderson BO, Repine JE. Circulating xanthine oxidase mediates lung neutrophil sequestration after intestinal ischemia-reperfusion. Am J Physiol 1992;263:L394-401.
412. Fialkow L, Wang Y, Downey GP. Reactive oxygen and nitrogen species as signaling molecules regulating neutrophil function. Free Radic Biol Med 2007;42:153-64.
413. Fialkow L, Chan CK, Grinstein S, Downey GP. Regulation of tyrosine phosphorylation in neutrophils by the NADPH oxidase. Role of reactive oxygen intermediates. J Biol Chem 1993;268:17131-7.
414. Fialkow L, Chan CK, Downey GP. Inhibition of CD45 during neutrophil activation. J Immunol 1997;158:5409-17.
415. Gadek JE, Pacht ER. The interdependence of lung antioxidants and antiprotease defense in ARDS. Chest 1996;110:273S-277S.
416. Haddad JJ, Olver RE, Land SC. Antioxidant/pro-oxidant equilibrium regulates HIF-1alpha and NF-kappa B redox sensitivity. Evidence for inhibition by glutathione oxidation in alveolar epithelial cells. J Biol Chem 2000;275:21130-9.
417. Quinlan GJ, Lamb NJ, Tilley R, Evans TW, Gutteridge JM. Plasma hypoxanthine levels in ARDS: implications for oxidative stress, morbidity, and mortality. Am J Respir Crit Care Med 1997;155:479-84.

418. Baldwin SR, Simon RH, Grum CM, Ketai LH, Boxer LA, Devall LJ. Oxidant activity in expired breath of patients with adult respiratory distress syndrome. Lancet 1986;1:11-4.
419. Xu J, Xie Z, Reece R, Pimental D, Zou MH. Uncoupling of endothelial nitric oxidase synthase by hypochlorous acid: role of NAD(P)H oxidase-derived superoxide and peroxynitrite. Arterioscler Thromb Vasc Biol 2006;26:2688-95.
420. Gole MD, Souza JM, Choi I, Hertkorn C, Malcolm S, Foust RF, 3rd, Finkel B, Lanken PN, Ischiropoulos H. Plasma proteins modified by tyrosine nitration in acute respiratory distress syndrome. Am J Physiol Lung Cell Mol Physiol 2000;278:L961-7.
421. Sittipunt C, Steinberg KP, Ruzinski JT, Myles C, Zhu S, Goodman RB, Hudson LD, Matalon S, Martin TR. Nitric oxide and nitrotyrosine in the lungs of patients with acute respiratory distress syndrome. Am J Respir Crit Care Med 2001;163:503-10.
422. Pacht ER, Timerman AP, Lykens MG, Merola AJ. Deficiency of alveolar fluid glutathione in patients with sepsis and the adult respiratory distress syndrome. Chest 1991;100:1397-403.
423. Lenz AG, Jorens PG, Meyer B, De Backer W, Van Overveld F, Bossaert L, Maier KL. Oxidatively modified proteins in bronchoalveolar lavage fluid of patients with ARDS and patients at-risk for ARDS. Eur Respir J 1999;13:169-74.
424. Carpenter CT, Price PV, Christman BW. Exhaled breath condensate isoprostanes are elevated in patients with acute lung injury or ARDS. Chest 1998;114:1653-9.
425. Brigham KL. Role of free radicals in lung injury. Chest 1986;89:859-63.
426. Babior BM, Lambeth JD, Nauseef W. The neutrophil NADPH oxidase. Arch Biochem Biophys 2002;397:342-4.
427. Bogdan C. Nitric oxide and the immune response. Nat Immunol 2001;2:907-16.
428. Albrich JM, McCarthy CA, Hurst JK. Biological reactivity of hypochlorous acid: implications for microbicidal mechanisms of leukocyte myeloperoxidase. Proc Natl Acad Sci U S A 1981;78:210-4.
429. Malle E, Marsche G, Panzenboeck U, Sattler W. Myeloperoxidase-mediated oxidation of high-density lipoproteins: fingerprints of newly recognized potential proatherogenic lipoproteins. Arch Biochem Biophys 2006;445:245-55.
430. Rey FE, Li XC, Carretero OA, Garvin JL, Pagano PJ. Perivascular superoxide anion contributes to impairment of endothelium-dependent relaxation: role of gp91(phox). Circulation 2002;106:2497-502.
431. Banfi B, Clark RA, Steger K, Krause KH. Two novel proteins activate superoxide generation by the NADPH oxidase NOX1. J Biol Chem 2003;278:3510-3.
432. Sorescu D, Weiss D, Lassegue B, Clempus RE, Szocs K, Sorescu GP, Valppu L, Quinn MT, Lambeth JD, Vega JD, Taylor WR, Griendling KK. Superoxide production and expression of nox family proteins in human atherosclerosis. Circulation 2002;105:1429-35.
433. Kirkinezos IG, Bacman SR, Hernandez D, Oca-Cossio J, Arias LJ, Perez-Pinzon MA, Bradley WG, Moraes CT. Cytochrome c association with the inner mitochondrial membrane is impaired in the CNS of G93A-SOD1 mice. J Neurosci 2005;25:164-72.

434. Nieto N, Friedman SL, Cederbaum AI. Cytochrome P450 2E1-derived reactive oxygen species mediate paracrine stimulation of collagen I protein synthesis by hepatic stellate cells. J Biol Chem 2002;277:9853-64.
435. Meneshian A, Bulkley GB. The physiology of endothelial xanthine oxidase: from urate catabolism to reperfusion injury to inflammatory signal transduction. Microcirculation 2002;9:161-75.
436. Shvedova AA, Tyurina JY, Kawai K, Tyurin VA, Kommineni C, Castranova V, Fabisiak JP, Kagan VE. Selective peroxidation and externalization of phosphatidylserine in normal human epidermal keratinocytes during oxidative stress induced by cumene hydroperoxide. J Invest Dermatol 2002;118:1008-18.
437. Feldhaus MJ, Weyrich AS, Zimmerman GA, McIntyre TM. Ceramide generation in situ alters leukocyte cytoskeletal organization and beta 2-integrin function and causes complete degranulation. J Biol Chem 2002;277:4285-93.
438. Schwartz MD, Moore EE, Moore FA, Shenkar R, Moine P, Haenel JB, Abraham E. Nuclear factor-kappa B is activated in alveolar macrophages from patients with acute respiratory distress syndrome. Crit Care Med 1996;24:1285-92.
439. Le Tulzo Y, Shenkar R, Kaneko D, Moine P, Fantuzzi G, Dinarello CA, Abraham E. Hemorrhage increases cytokine expression in lung mononuclear cells in mice: involvement of catecholamines in nuclear factor-kappaB regulation and cytokine expression. J Clin Invest 1997;99:1516-24.
440. Moine P, Shenkar R, Kaneko D, Le Tulzo Y, Abraham E. Systemic blood loss affects NF-kappa B regulatory mechanisms in the lungs. Am J Physiol 1997;273:L185-92.
441. Bowler RP, Velsor LW, Duda B, Chan ED, Abraham E, Ware LB, Matthay MA, Day BJ. Pulmonary edema fluid antioxidants are depressed in acute lung injury. Crit Care Med 2003;31:2309-15.
442. Cochrane CG, Spragg R, Revak SD. Pathogenesis of the adult respiratory distress syndrome. Evidence of oxidant activity in bronchoalveolar lavage fluid. J Clin Invest 1983;71:754-61.
443. Tasaka S, Amaya F, Hashimoto S, Ishizaka A. Roles of oxidants and redox signaling in the pathogenesis of acute respiratory distress syndrome. Antioxid Redox Signal 2008;10:739-53.
444. Kelly FJ, Mudway IS. Protein oxidation at the air-lung interface. Amino Acids 2003;25:375-96.
445. Lenz AG, Costabel U, Maier KL. Oxidized BAL fluid proteins in patients with interstitial lung diseases. Eur Respir J 1996;9:307-12.
446. Carr AC, Winterbourn CC. Oxidation of neutrophil glutathione and protein thiols by myeloperoxidase-derived hypochlorous acid. Biochem J 1997;327 (Pt 1):275-81.
447. Kulcharyk PA, Heinecke JW. Hypochlorous acid produced by the myeloperoxidase system of human phagocytes induces covalent cross-links between DNA and protein. Biochemistry 2001;40:3648-56.
448. Martin C, Papazian L, Payan MJ, Saux P, Gouin F. Pulmonary fibrosis correlates with outcome in adult respiratory distress syndrome. A study in mechanically ventilated patients. Chest 1995;107:196-200.
449. Martinet Y, Menard O, Vaillant P, Vignaud JM, Martinet N. Cytokines in human lung fibrosis. Arch Toxicol Suppl 1996;18:127-39.

450. Lindroos PM, Coin PG, Osornio-Vargas AR, Bonner JC. Interleukin 1 beta (IL-1 beta) and the IL-1 beta-alpha 2-macroglobulin complex upregulate the platelet-derived growth factor alpha-receptor on rat pulmonary fibroblasts. Am J Respir Cell Mol Biol 1995;13:455-65.
451. Bregeon F, Delpierre S, Chetaille B, Kajikawa O, Martin TR, Autillo-Touati A, Jammes Y, Pugin J. Mechanical ventilation affects lung function and cytokine production in an experimental model of endotoxemia. Anesthesiology 2005;102:331-9.
452. Bregeon F, Roch A, Delpierre S, Ghigo E, Autillo-Touati A, Kajikawa O, Martin TR, Pugin J, Portugal H, Auffray JP, Jammes Y. Conventional mechanical ventilation of healthy lungs induced pro-inflammatory cytokine gene transcription. Respir Physiol Neurobiol 2002;132:191-203.
453. Madtes DK, Rubenfeld G, Klima LD, Milberg JA, Steinberg KP, Martin TR, Raghu G, Hudson LD, Clark JG. Elevated transforming growth factor-alpha levels in bronchoalveolar lavage fluid of patients with acute respiratory distress syndrome. Am J Respir Crit Care Med 1998;158:424-30.
454. Kheradmand F, Folkesson HG, Shum L, Derynk R, Pytela R, Matthay MA. Transforming growth factor-alpha enhances alveolar epithelial cell repair in a new in vitro model. Am J Physiol 1994;267:L728-38.
455. Verghese GM, McCormick-Shannon K, Mason RJ, Matthay MA. Hepatocyte growth factor and keratinocyte growth factor in the pulmonary edema fluid of patients with acute lung injury. Biologic and clinical significance. Am J Respir Crit Care Med 1998;158:386-94.
456. Fowler AA, Hamman RF, Zerbe GO, Benson KN, Hyers TM. Adult respiratory distress syndrome. Prognosis after onset. Am Rev Respir Dis 1985;132:472-8.
457. Suchyta MR, Clemmer TP, Elliott CG, Orme JF, Jr., Weaver LK. The adult respiratory distress syndrome. A report of survival and modifying factors. Chest 1992;101:1074-9.
458. Dreyfuss D, Soler P, Saumon G. Mechanical ventilation-induced pulmonary edema. Interaction with previous lung alterations. Am J Respir Crit Care Med 1995;151:1568-75.
459. Dreyfuss D, Soler P, Saumon G. Spontaneous resolution of pulmonary edema caused by short periods of cyclic overinflation. J Appl Physiol 1992;72:2081-9.
460. Kacmarek RM. Ventilator-associated lung injury. Int Anesthesiol Clin 1999;37:47-64.
461. Altemeier WA, Matute-Bello G, Frevert CW, Kawata Y, Kajikawa O, Martin TR, Glenny RW. Mechanical ventilation with moderate tidal volumes synergistically increases lung cytokine response to systemic endotoxin. Am J Physiol Lung Cell Mol Physiol 2004;287:L533-42.
462. Nash G, Blennerhassett J, Pontoppidan H. Pulmonary lesions associated with oxygen therapy and artificial ventilation. Laval Med 1968;39:59-64.
463. Nash G, Blennerhassett JB, Pontoppidan H. Pulmonary lesions associated with oxygen therapy and artifical ventilation. N Engl J Med 1967;276:368-74.
464. Tremblay L, Valenza F, Ribeiro SP, Li J, Slutsky AS. Injurious ventilatory strategies increase cytokines and c-fos m-RNA expression in an isolated rat lung model. J Clin Invest 1997;99:944-52.
465. Tremblay LN, Miatto D, Hamid Q, Govindarajan A, Slutsky AS. Injurious ventilation induces widespread pulmonary epithelial expression of tumor

necrosis factor-alpha and interleukin-6 messenger RNA. Crit Care Med 2002;30:1693-700.
466. Ranieri VM, Giunta F, Suter PM, Slutsky AS. Mechanical ventilation as a mediator of multisystem organ failure in acute respiratory distress syndrome. JAMA 2000;284:43-4.
467. Hashimoto S, Kobayashi A, Kooguchi K, Kitamura Y, Onodera H, Nakajima H. Upregulation of two death pathways of perforin/granzyme and FasL/Fas in septic acute respiratory distress syndrome. Am J Respir Crit Care Med 2000;161:237-43.
468. Albertine KH, Soulier MF, Wang Z, Ishizaka A, Hashimoto S, Zimmerman GA, Matthay MA, Ware LB. Fas and fas ligand are up-regulated in pulmonary edema fluid and lung tissue of patients with acute lung injury and the acute respiratory distress syndrome. Am J Pathol 2002;161:1783-96.
469. Dosreis GA, Borges VM, Zin WA. The central role of Fas-ligand cell signaling in inflammatory lung diseases. J Cell Mol Med 2004;8:285-93.
470. Matute-Bello G, Frevert CW, Liles WC, Nakamura M, Ruzinski JT, Ballman K, Wong VA, Vathanaprida C, Martin TR. Fas/Fas ligand system mediates epithelial injury, but not pulmonary host defenses, in response to inhaled bacteria. Infect Immun 2001;69:5768-76.
471. Mizuta M, Nakajima H, Mizuta N, Kitamura Y, Nakajima Y, Hashimoto S, Matsuyama H, Shime N, Amaya F, Koh H, Ishizaka A, Magae J, Tanuma SI. Fas ligand released by activated monocytes causes apoptosis of lung epithelial cells in human acute lung injury model in vitro. Biol Pharm Bull 2008;31:386-90.
472. Neff TA, Guo RF, Neff SB, Sarma JV, Speyer CL, Gao H, Bernacki KD, Huber-Lang M, McGuire S, Hoesel LM, Riedemann NC, Beck-Schimmer B, Zetoune FS, Ward PA. Relationship of acute lung inflammatory injury to Fas/FasL system. Am J Pathol 2005;166:685-94.
473. DosReis GA, Borges VM. Role of Fas-ligand induced apoptosis in pulmonary inflammation and injury. Curr Drug Targets Inflamm Allergy 2003;2:161-7.
474. Levine SJ. Fas-mediated alveolar epithelial cell apoptosis in acute lung injury: friend or foe? Crit Care Med 2002;30:1667-8.
475. Wang HC, Shun CT, Hsu SM, Kuo SH, Luh KT, Yang PC. Fas/Fas ligand pathway is involved in the resolution of type II pneumocyte hyperplasia after acute lung injury: evidence from a rat model. Crit Care Med 2002;30:1528-34.
476. Kuwano K, Miyazaki H, Hagimoto N, Kawasaki M, Fujita M, Kunitake R, Kaneko Y, Hara N. The involvement of Fas-Fas ligand pathway in fibrosing lung diseases. Am J Respir Cell Mol Biol 1999;20:53-60.
477. Maeyama T, Kuwano K, Kawasaki M, Kunitake R, Hagimoto N, Matsuba T, Yoshimi M, Inoshima I, Yoshida K, Hara N. Upregulation of Fas-signalling molecules in lung epithelial cells from patients with idiopathic pulmonary fibrosis. Eur Respir J 2001;17:180-9.
478. Suda T, Takahashi T, Golstein P, Nagata S. Molecular cloning and expression of the Fas ligand, a novel member of the tumor necrosis factor family. Cell 1993;75:1169-78.
479. Itoh N, Yonehara S, Ishii A, Yonehara M, Mizushima S, Sameshima M, Hase A, Seto Y, Nagata S. The polypeptide encoded by the cDNA for human cell surface antigen Fas can mediate apoptosis. Cell 1991;66:233-43.

480. Nagata S. Fas and Fas ligand: a death factor and its receptor. Adv Immunol 1994;57:129-44.
481. Suda T, Hashimoto H, Tanaka M, Ochi T, Nagata S. Membrane Fas ligand kills human peripheral blood T lymphocytes, and soluble Fas ligand blocks the killing. J Exp Med 1997;186:2045-50.
482. Tanaka M, Itai T, Adachi M, Nagata S. Downregulation of Fas ligand by shedding. Nat Med 1998;4:31-6.
483. Kayagaki N, Kawasaki A, Ebata T, Ohmoto H, Ikeda S, Inoue S, Yoshino K, Okumura K, Yagita H. Metalloproteinase-mediated release of human Fas ligand. J Exp Med 1995;182:1777-83.
484. Kayagaki N, Yagita H. [Metalloproteinase-mediated release of human fas ligand]. Nippon Rinsho 1996;54:1747-52.
485. Knox PG, Milner AE, Green NK, Eliopoulos AG, Young LS. Inhibition of metalloproteinase cleavage enhances the cytotoxicity of Fas ligand. J Immunol 2003;170:677-85.
486. Mitsiades N, Yu WH, Poulaki V, Tsokos M, Stamenkovic I. Matrix metalloproteinase-7-mediated cleavage of Fas ligand protects tumor cells from chemotherapeutic drug cytotoxicity. Cancer Res 2001;61:577-81.
487. Powell WC, Fingleton B, Wilson CL, Boothby M, Matrisian LM. The metalloproteinase matrilysin proteolytically generates active soluble Fas ligand and potentiates epithelial cell apoptosis. Curr Biol 1999;9:1441-7.
488. Vargo-Gogola T, Crawford HC, Fingleton B, Matrisian LM. Identification of novel matrix metalloproteinase-7 (matrilysin) cleavage sites in murine and human Fas ligand. Arch Biochem Biophys 2002;408:155-61.
489. Hosaka N, Oyaizu N, Kaplan MH, Yagita H, Pahwa S. Membrane and soluble forms of Fas (CD95) and Fas ligand in peripheral blood mononuclear cells and in plasma from human immunodeficiency virus-infected persons. J Infect Dis 1998;178:1030-9.
490. Kiener PA, Davis PM, Rankin BM, Klebanoff SJ, Ledbetter JA, Starling GC, Liles WC. Human monocytic cells contain high levels of intracellular Fas ligand: rapid release following cellular activation. J Immunol 1997;159:1594-8.
491. Liles WC, Kiener PA, Ledbetter JA, Aruffo A, Klebanoff SJ. Differential expression of Fas (CD95) and Fas ligand on normal human phagocytes: implications for the regulation of apoptosis in neutrophils. J Exp Med 1996;184:429-40.
492. Tanaka M, Suda T, Takahashi T, Nagata S. Expression of the functional soluble form of human fas ligand in activated lymphocytes. EMBO J 1995;14:1129-35.
493. Lowin B, Mattman C, Hahne M, Tschopp J. Comparison of Fas(Apo-1/CD95)- and perforin-mediated cytotoxicity in primary T lymphocytes. Int Immunol 1996;8:57-63.
494. Kagi D, Vignaux F, Ledermann B, Burki K, Depraetere V, Nagata S, Hengartner H, Golstein P. Fas and perforin pathways as major mechanisms of T cell-mediated cytotoxicity. Science 1994;265:528-30.
495. Dhein J, Walczak H, Baumler C, Debatin KM, Krammer PH. Autocrine T-cell suicide mediated by APO-1/(Fas/CD95). Nature 1995;373:438-41.
496. Cheng J, Zhou T, Liu C, Shapiro JP, Brauer MJ, Kiefer MC, Barr PJ, Mountz JD. Protection from Fas-mediated apoptosis by a soluble form of the Fas molecule. Science 1994;263:1759-62.

497. Nakamura M, Matute-Bello G, Liles WC, Hayashi S, Kajikawa O, Lin SM, Frevert CW, Martin TR. Differential response of human lung epithelial cells to fas-induced apoptosis. Am J Pathol 2004;164:1949-58.
498. Oyaizu N, Adachi Y, Hashimoto F, McCloskey TW, Hosaka N, Kayagaki N, Yagita H, Pahwa S. Monocytes express Fas ligand upon CD4 cross-linking and induce CD4+ T cells apoptosis: a possible mechanism of bystander cell death in HIV infection. J Immunol 1997;158:2456-63.
499. Park DR, Thomsen AR, Frevert CW, Pham U, Skerrett SJ, Kiener PA, Liles WC. Fas (CD95) induces proinflammatory cytokine responses by human monocytes and monocyte-derived macrophages. J Immunol 2003;170:6209-16.
500. Abraham E, Baughman R, Fletcher E, Heard S, Lamberti J, Levy H, Nelson L, Rumbak M, Steingrub J, Taylor J, Park YC, Hynds JM, Freitag J. Liposomal prostaglandin E1 (TLC C-53) in acute respiratory distress syndrome: a controlled, randomized, double-blind, multicenter clinical trial. TLC C-53 ARDS Study Group. Crit Care Med 1999;27:1478-85.
501. Boussaud V, Soler P, Moreau J, Goodwin RG, Hance AJ. Expression of three members of the TNF-R family of receptors (4-1BB, lymphotoxin-beta receptor, and Fas) in human lung. Eur Respir J 1998;12:926-31.
502. Hamann KJ, Dorscheid DR, Ko FD, Conforti AE, Sperling AI, Rabe KF, White SR. Expression of Fas (CD95) and FasL (CD95L) in human airway epithelium. Am J Respir Cell Mol Biol 1998;19:537-42.
503. Rafi AQ, Zeytun A, Bradley MJ, Sponenberg DP, Grayson RL, Nagarkatti M, Nagarkatti PS. Evidence for the involvement of Fas ligand and perforin in the induction of vascular leak syndrome. J Immunol 1998;161:3077-86.
504. Arase H, Arase N, Saito T. Fas-mediated cytotoxicity by freshly isolated natural killer cells. J Exp Med 1995;181:1235-8.
505. Nagafuji K, Shibuya T, Harada M, Mizuno S, Takenaka K, Miyamoto T, Okamura T, Gondo H, Niho Y. Functional expression of Fas antigen (CD95) on hematopoietic progenitor cells. Blood 1995;86:883-9.
506. Rouvier E, Luciani MF, Golstein P. Fas involvement in Ca(2+)-independent T cell-mediated cytotoxicity. J Exp Med 1993;177:195-200.
507. Iwamoto M, Koji T, Makiyama K, Kobayashi N, Nakane PK. Apoptosis of crypt epithelial cells in ulcerative colitis. J Pathol 1996;180:152-9.
508. Lynch DH, Ramsdell F, Alderson MR. Fas and FasL in the homeostatic regulation of immune responses. Immunol Today 1995;16:569-74.
509. Lynch DH, Alderson MR, Ramsdell F. Immunoregulatory effects of Fas-mediated signalling. J Cell Biochem 1996;60:39-46.
510. Allison J, Georgiou HM, Strasser A, Vaux DL. Transgenic expression of CD95 ligand on islet beta cells induces a granulocytic infiltration but does not confer immune privilege upon islet allografts. Proc Natl Acad Sci U S A 1997;94:3943-7.
511. Rensing-Ehl A, Frei K, Flury R, Matiba B, Mariani SM, Weller M, Aebischer P, Krammer PH, Fontana A. Local Fas/APO-1 (CD95) ligand-mediated tumor cell killing in vivo. Eur J Immunol 1995;25:2253-8.
512. Hunt JS, Vassmer D, Ferguson TA, Miller L. Fas ligand is positioned in mouse uterus and placenta to prevent trafficking of activated leukocytes between the mother and the conceptus. J Immunol 1997;158:4122-8.

513. French LE, Tschopp J. Constitutive Fas ligand expression in several non-lymphoid mouse tissues: implications for immune-protection and cell turnover. Behring Inst Mitt 1996:156-60.
514. Micheau O, Thome M, Schneider P, Holler N, Tschopp J, Nicholson DW, Briand C, Grutter MG. The long form of FLIP is an activator of caspase-8 at the Fas death-inducing signaling complex. J Biol Chem 2002;277:45162-71.
515. Thome M, Tschopp J. Regulation of lymphocyte proliferation and death by FLIP. Nat Rev Immunol 2001;1:50-8.
516. Kischkel FC, Lawrence DA, Chuntharapai A, Schow P, Kim KJ, Ashkenazi A. Apo2L/TRAIL-dependent recruitment of endogenous FADD and caspase-8 to death receptors 4 and 5. Immunity 2000;12:611-20.
517. Siegel RM, Frederiksen JK, Zacharias DA, Chan FK, Johnson M, Lynch D, Tsien RY, Lenardo MJ. Fas preassociation required for apoptosis signaling and dominant inhibition by pathogenic mutations. Science 2000;288:2354-7.
518. Fanzo JC, Lynch MP, Phee H, Hyer M, Cremesti A, Grassme H, Norris JS, Coggeshall KM, Rueda BR, Pernis AB, Kolesnick R, Gulbins E. CD95 rapidly clusters in cells of diverse origins. Cancer Biol Ther 2003;2:392-5.
519. Algeciras-Schimnich A, Shen L, Barnhart BC, Murmann AE, Burkhardt JK, Peter ME. Molecular ordering of the initial signaling events of CD95. Mol Cell Biol 2002;22:207-20.
520. Peter ME, Krammer PH. The CD95(APO-1/Fas) DISC and beyond. Cell Death Differ 2003;10:26-35.
521. Lee KH, Feig C, Tchikov V, Schickel R, Hallas C, Schutze S, Peter ME, Chan AC. The role of receptor internalization in CD95 signaling. EMBO J 2006;25:1009-23.
522. Siegel RM, Muppidi JR, Sarker M, Lobito A, Jen M, Martin D, Straus SE, Lenardo MJ. SPOTS: signaling protein oligomeric transduction structures are early mediators of death receptor-induced apoptosis at the plasma membrane. J Cell Biol 2004;167:735-44.
523. Tornusciolo DR, Schmidt RE, Roth KA. Simultaneous detection of TDT-mediated dUTP-biotin nick end-labeling (TUNEL)-positive cells and multiple immunohistochemical markers in single tissue sections. Biotechniques 1995;19:800-5.
524. Konopleva M, Zhao S, Xie Z, Segall H, Younes A, Claxton DF, Estrov Z, Kornblau SM, Andreeff M. Apoptosis. Molecules and mechanisms. Adv Exp Med Biol 1999;457:217-36.
525. Deveraux QL, Reed JC. IAP family proteins--suppressors of apoptosis. Genes Dev 1999;13:239-52.
526. Deveraux QL, Roy N, Stennicke HR, Van Arsdale T, Zhou Q, Srinivasula SM, Alnemri ES, Salvesen GS, Reed JC. IAPs block apoptotic events induced by caspase-8 and cytochrome c by direct inhibition of distinct caspases. EMBO J 1998;17:2215-23.
527. Deveraux QL, Schendel SL, Reed JC. Antiapoptotic proteins. The bcl-2 and inhibitor of apoptosis protein families. Cardiol Clin 2001;19:57-74.
528. Tschopp J, Irmler M, Thome M. Inhibition of fas death signals by FLIPs. Curr Opin Immunol 1998;10:552-8.

529. Bannerman DD, Eiting KT, Winn RK, Harlan JM. FLICE-like inhibitory protein (FLIP) protects against apoptosis and suppresses NF-kappaB activation induced by bacterial lipopolysaccharide. Am J Pathol 2004;165:1423-31.
530. Bratton DL, Fadok VA, Richter DA, Kailey JM, Guthrie LA, Henson PM. Appearance of phosphatidylserine on apoptotic cells requires calcium-mediated nonspecific flip-flop and is enhanced by loss of the aminophospholipid translocase. J Biol Chem 1997;272:26159-65.
531. Fadok VA, de Cathelineau A, Daleke DL, Henson PM, Bratton DL. Loss of phospholipid asymmetry and surface exposure of phosphatidylserine is required for phagocytosis of apoptotic cells by macrophages and fibroblasts. J Biol Chem 2001;276:1071-7.
532. O'Neill S, O'Neill AJ, Conroy E, Brady HR, Fitzpatrick JM, Watson RW. Altered caspase expression results in delayed neutrophil apoptosis in acute pancreatitis. J Leukoc Biol 2000;68:15-20.
533. Kataoka T, Budd RC, Holler N, Thome M, Martinon F, Irmler M, Burns K, Hahne M, Kennedy N, Kovacsovics M, Tschopp J. The caspase-8 inhibitor FLIP promotes activation of NF-kappaB and Erk signaling pathways. Curr Biol 2000;10:640-8.
534. Ponton A, Clement MV, Stamenkovic I. The CD95 (APO-1/Fas) receptor activates NF-kappaB independently of its cytotoxic function. J Biol Chem 1996;271:8991-5.
535. Rensing-Ehl A, Hess S, Ziegler-Heitbrock HW, Riethmuller G, Engelmann H. Fas/Apo-1 activates nuclear factor kappa B and induces interleukin-6 production. J Inflamm 1995;45:161-74.
536. Kataoka T, Tschopp J. N-terminal fragment of c-FLIP(L) processed by caspase 8 specifically interacts with TRAF2 and induces activation of the NF-kappaB signaling pathway. Mol Cell Biol 2004;24:2627-36.
537. Ahn JH, Park SM, Cho HS, Lee MS, Yoon JB, Vilcek J, Lee TH. Non-apoptotic signaling pathways activated by soluble Fas ligand in serum-starved human fibroblasts. Mitogen-activated protein kinases and NF-kappaB-dependent gene expression. J Biol Chem 2001;276:47100-6.
538. Dupont PJ, Warrens AN. Fas ligand exerts its pro-inflammatory effects via neutrophil recruitment but not activation. Immunology 2007;120:133-9.
539. Hagimoto N, Kuwano K, Kawasaki M, Yoshimi M, Kaneko Y, Kunitake R, Maeyama T, Tanaka T, Hara N. Induction of interleukin-8 secretion and apoptosis in bronchiolar epithelial cells by Fas ligation. Am J Respir Cell Mol Biol 1999;21:436-45.
540. Imamura R, Konaka K, Matsumoto N, Hasegawa M, Fukui M, Mukaida N, Kinoshita T, Suda T. Fas ligand induces cell-autonomous NF-kappaB activation and interleukin-8 production by a mechanism distinct from that of tumor necrosis factor-alpha. J Biol Chem 2004;279:46415-23.
541. Hashimoto F, Oyaizu N, Kalyanaraman VS, Pahwa S. Modulation of Bcl-2 protein by CD4 cross-linking: a possible mechanism for lymphocyte apoptosis in human immunodeficiency virus infection and for rescue of apoptosis by interleukin-2. Blood 1997;90:745-53.
542. Kuwano K, Hagimoto N, Kawasaki M, Nakamura N, Shirakawa K, Maeyama T, Hara N. Expression of FasL and Fas protein and their soluble form in patients with hypersensitivity pneumonitis. Int Arch Allergy Immunol 2000;122:209-15.

543. Imai Y, Parodo J, Kajikawa O, de Perrot M, Fischer S, Edwards V, Cutz E, Liu M, Keshavjee S, Martin TR, Marshall JC, Ranieri VM, Slutsky AS. Injurious mechanical ventilation and end-organ epithelial cell apoptosis and organ dysfunction in an experimental model of acute respiratory distress syndrome. JAMA 2003;289:2104-12.
544. Iwama H, Tohma J, Nakamura N. High serum soluble Fas-ligand in cardiopulmonary arrest patients. Am J Emerg Med 2000;18:348.
545. Perianayagam MC, Murray SL, Balakrishnan VS, Guo D, King AJ, Pereira BJ, Jaber BL. Serum soluble Fas (CD95) and Fas ligand profiles in chronic kidney failure. J Lab Clin Med 2000;136:320-7.
546. Doughty L, Clark RS, Kaplan SS, Sasser H, Carcillo J. sFas and sFas ligand and pediatric sepsis-induced multiple organ failure syndrome. Pediatr Res 2002;52:922-7.
547. Kitamura M, Endo S, Sato N, Yaegashi Y, Suxzuki Y, Kojika N, Wakabayashi G. Soluble Fas and soluble FasL in multiple organ dysfunction syndrome complicating diffuse peritonitis. Res Commun Mol Pathol Pharmacol 2004;115-116:235-42.
548. Matute-Bello G, Frevert CW, Martin TR. Animal models of acute lung injury. Am J Physiol Lung Cell Mol Physiol 2008;295:L379-99.
549. Matute-Bello G, Liles WC, Frevert CW, Nakamura M, Ballman K, Vathanaprida C, Kiener PA, Martin TR. Recombinant human Fas ligand induces alveolar epithelial cell apoptosis and lung injury in rabbits. Am J Physiol Lung Cell Mol Physiol 2001;281:L328-35.
550. Perl M, Chung CS, Perl U, Lomas-Neira J, de Paepe M, Cioffi WG, Ayala A. Fas-induced pulmonary apoptosis and inflammation during indirect acute lung injury. Am J Respir Crit Care Med 2007;176:591-601.
551. Tateda K, Deng JC, Moore TA, Newstead MW, Paine R, 3rd, Kobayashi N, Yamaguchi K, Standiford TJ. Hyperoxia mediates acute lung injury and increased lethality in murine Legionella pneumonia: the role of apoptosis. J Immunol 2003;170:4209-16.
552. Matthay MA, Folkesson HG, Campagna A, Kheradmand F. Alveolar epithelial barrier and acute lung injury. New Horiz 1993;1:613-22.
553. Mantell LL, Horowitz S, Davis JM, Kazzaz JA. Hyperoxia-induced cell death in the lung--the correlation of apoptosis, necrosis, and inflammation. Ann N Y Acad Sci 1999;887:171-80.
554. Peter ME, Budd RC, Desbarats J, Hedrick SM, Hueber AO, Newell MK, Owen LB, Pope RM, Tschopp J, Wajant H, Wallach D, Wiltrout RH, Zornig M, Lynch DH. The CD95 receptor: apoptosis revisited. Cell 2007;129:447-50.
555. Matute-Bello G, Lee JS, Liles WC, Frevert CW, Mongovin S, Wong V, Ballman K, Sutlief S, Martin TR. Fas-mediated acute lung injury requires fas expression on nonmyeloid cells of the lung. J Immunol 2005;175:4069-75.
556. Matute-Bello G, Liles WC, Frevert CW, Dhanireddy S, Ballman K, Wong V, Green RR, Song HY, Witcher DR, Jakubowski JA, Martin TR. Blockade of the Fas/FasL system improves pneumococcal clearance from the lungs without preventing dissemination of bacteria to the spleen. J Infect Dis 2005;191:596-606.

557. Holmes MC, Zhang P, Nelson S, Summer WR, Bagby GJ. Neutrophil modulation of the pulmonary chemokine response to lipopolysaccharide. Shock 2002;18:555-60.
558. Grassme H, Kirschnek S, Riethmueller J, Riehle A, von Kurthy G, Lang F, Weller M, Gulbins E. CD95/CD95 ligand interactions on epithelial cells in host defense to Pseudomonas aeruginosa. Science 2000;290:527-30.
559. Schaub FJ, Han DK, Liles WC, Adams LD, Coats SA, Ramachandran RK, Seifert RA, Schwartz SM, Bowen-Pope DF. Fas/FADD-mediated activation of a specific program of inflammatory gene expression in vascular smooth muscle cells. Nat Med 2000;6:790-6.
560. Wortinger MA, Foley JW, Larocque P, Witcher DR, Lahn M, Jakubowski JA, Glasebrook A, Song HY. Fas ligand-induced murine pulmonary inflammation is reduced by a stable decoy receptor 3 analogue. Immunology 2003;110:225-33.
561. Matute-Bello G, Winn RK, Martin TR, Liles WC. Sustained lipopolysaccharide-induced lung inflammation in mice is attenuated by functional deficiency of the Fas/Fas ligand system. Clin Diagn Lab Immunol 2004;11:358-61.
562. Neff SB, Z'Graggen B R, Neff TA, Jamnicki-Abegg M, Suter D, Schimmer RC, Booy C, Joch H, Pasch T, Ward PA, Beck-Schimmer B. Inflammatory response of tracheobronchial epithelial cells to endotoxin. Am J Physiol Lung Cell Mol Physiol 2006;290:L86-96.
563. Matsuda N, Yamamoto S, Takano K, Kageyama S, Kurobe Y, Yoshihara Y, Takano Y, Hattori Y. Silencing of fas-associated death domain protects mice from septic lung inflammation and apoptosis. Am J Respir Crit Care Med 2009;179:806-15.
564. Guo RF, Ward PA. Mediators and regulation of neutrophil accumulation in inflammatory responses in lung: insights from the IgG immune complex model. Free Radic Biol Med 2002;33:303-10.
565. Hohlbaum AM, Gregory MS, Ju ST, Marshak-Rothstein A. Fas ligand engagement of resident peritoneal macrophages in vivo induces apoptosis and the production of neutrophil chemotactic factors. J Immunol 2001;167:6217-24.
566. Bem RA, Farnand AW, Wong V, Koski A, Rosenfeld ME, van Rooijen N, Frevert CW, Martin TR, Matute-Bello G. Depletion of resident alveolar macrophages does not prevent Fas-mediated lung injury in mice. Am J Physiol Lung Cell Mol Physiol 2008;295:L314-25.
567. Perl M, Chung CS, Lomas-Neira J, Rachel TM, Biffl WL, Cioffi WG, Ayala A. Silencing of Fas, but not caspase-8, in lung epithelial cells ameliorates pulmonary apoptosis, inflammation, and neutrophil influx after hemorrhagic shock and sepsis. Am J Pathol 2005;167:1545-59.
568. Crestani B, Cornillet P, Dehoux M, Rolland C, Guenounou M, Aubier M. Alveolar type II epithelial cells produce interleukin-6 in vitro and in vivo. Regulation by alveolar macrophage secretory products. J Clin Invest 1994;94:731-40.
569. DiMango E, Zar HJ, Bryan R, Prince A. Diverse Pseudomonas aeruginosa gene products stimulate respiratory epithelial cells to produce interleukin-8. J Clin Invest 1995;96:2204-10.
570. Quesnel C, Marchand-Adam S, Fabre A, Marchal-Somme J, Philip I, Lasocki S, Lecon V, Crestani B, Dehoux M. Regulation of hepatocyte growth factor

secretion by fibroblasts in patients with acute lung injury. Am J Physiol Lung Cell Mol Physiol 2008;294:L334-43.
571. Thorley AJ, Ford PA, Giembycz MA, Goldstraw P, Young A, Tetley TD. Differential regulation of cytokine release and leukocyte migration by lipopolysaccharide-stimulated primary human lung alveolar type II epithelial cells and macrophages. J Immunol 2007;178:463-73.
572. Vanderbilt JN, Mager EM, Allen L, Sawa T, Wiener-Kronish J, Gonzalez R, Dobbs LG. CXC chemokines and their receptors are expressed in type II cells and upregulated following lung injury. Am J Respir Cell Mol Biol 2003;29:661-8.
573. Witherden IR, Vanden Bon EJ, Goldstraw P, Ratcliffe C, Pastorino U, Tetley TD. Primary human alveolar type II epithelial cell chemokine release: effects of cigarette smoke and neutrophil elastase. Am J Respir Cell Mol Biol 2004;30:500-9.
574. Fowler AA, Hyers TM, Fisher BJ, Bechard DE, Centor RM, Webster RO. The adult respiratory distress syndrome. Cell populations and soluble mediators in the air spaces of patients at high risk. Am Rev Respir Dis 1987;136:1225-31.
575. Bitterman PB, Polunovsky VA, Ingbar DH. Repair after acute lung injury. Chest 1994;105:118S-121S.
576. Polunovsky VA, Chen B, Henke C, Snover D, Wendt C, Ingbar DH, Bitterman PB. Role of mesenchymal cell death in lung remodeling after injury. J Clin Invest 1993;92:388-97.
577. Serrao KL, Fortenberry JD, Owens ML, Harris FL, Brown LA. Neutrophils induce apoptosis of lung epithelial cells via release of soluble Fas ligand. Am J Physiol Lung Cell Mol Physiol 2001;280:L298-305.
578. Olman MA, White KE, Ware LB, Simmons WL, Benveniste EN, Zhu S, Pugin J, Matthay MA. Pulmonary edema fluid from patients with early lung injury stimulates fibroblast proliferation through IL-1 beta-induced IL-6 expression. J Immunol 2004;172:2668-77.
579. Armstrong L, Thickett DR, Mansell JP, Ionescu M, Hoyle E, Billinghurst RC, Poole AR, Millar AB. Changes in collagen turnover in early acute respiratory distress syndrome. Am J Respir Crit Care Med 1999;160:1910-5.
580. Ichikado K, Suga M, Muranaka H, Gushima Y, Miyakawa H, Tsubamoto M, Johkoh T, Hirata N, Yoshinaga T, Kinoshita Y, Yamashita Y, Sasaki Y. Prediction of prognosis for acute respiratory distress syndrome with thin-section CT: validation in 44 cases. Radiology 2006;238:321-9.
581. Selman M, Pardo A. Idiopathic pulmonary fibrosis: an epithelial/fibroblastic cross-talk disorder. Respir Res 2002;3:3.
582. Selman M, Pardo A. Role of epithelial cells in idiopathic pulmonary fibrosis: from innocent targets to serial killers. Proc Am Thorac Soc 2006;3:364-72.
583. Selman M, Pardo A. Idiopathic pulmonary fibrosis: misunderstandings between epithelial cells and fibroblasts? Sarcoidosis Vasc Diffuse Lung Dis 2004;21:165-72.
584. Selman M, Pardo A. The epithelial/fibroblastic pathway in the pathogenesis of idiopathic pulmonary fibrosis. Am J Respir Cell Mol Biol 2003;29:S93-7.
585. Shetty S, Padijnayayveetil J, Tucker T, Stankowska D, Idell S. The fibrinolytic system and the regulation of lung epithelial cell proteolysis, signaling, and cellular viability. Am J Physiol Lung Cell Mol Physiol 2008;295:L967-75.

586. Kuwano K. Epithelial cell apoptosis and lung remodeling. Cell Mol Immunol 2007;4:419-29.
587. Plataki M, Koutsopoulos AV, Darivianaki K, Delides G, Siafakas NM, Bouros D. Expression of apoptotic and antiapoptotic markers in epithelial cells in idiopathic pulmonary fibrosis. Chest 2005;127:266-74.
588. Chapman HA. A Fas pathway to pulmonary fibrosis. J Clin Invest 1999;104:1-2.
589. Uhal BD, Joshi I, True AL, Mundle S, Raza A, Pardo A, Selman M. Fibroblasts isolated after fibrotic lung injury induce apoptosis of alveolar epithelial cells in vitro. Am J Physiol 1995;269:L819-28.
590. Uhal BD, Joshi I, Hughes WF, Ramos C, Pardo A, Selman M. Alveolar epithelial cell death adjacent to underlying myofibroblasts in advanced fibrotic human lung. Am J Physiol 1998;275:L1192-9.
591. Lee CG, Cho SJ, Kang MJ, Chapoval SP, Lee PJ, Noble PW, Yehualaeshet T, Lu B, Flavell RA, Milbrandt J, Homer RJ, Elias JA. Early growth response gene 1-mediated apoptosis is essential for transforming growth factor beta1-induced pulmonary fibrosis. J Exp Med 2004;200:377-89.
592. Shapiro SD, Campbell EJ, Senior RM, Welgus HG. Proteinases secreted by human mononuclear phagocytes. J Rheumatol Suppl 1991;27:95-8.
593. Gronski TJ, Jr., Martin RL, Kobayashi DK, Walsh BC, Holman MC, Huber M, Van Wart HE, Shapiro SD. Hydrolysis of a broad spectrum of extracellular matrix proteins by human macrophage elastase. J Biol Chem 1997;272:12189-94.
594. Orlinick JR, Elkon KB, Chao MV. Separate domains of the human fas ligand dictate self-association and receptor binding. J Biol Chem 1997;272:32221-9.
595. Orlinick JR, Vaishnaw A, Elkon KB, Chao MV. Requirement of cysteine-rich repeats of the Fas receptor for binding by the Fas ligand. J Biol Chem 1997;272:28889-94.
596. Vaishnaw AK, Orlinick JR, Chu JL, Krammer PH, Chao MV, Elkon KB. The molecular basis for apoptotic defects in patients with CD95 (Fas/Apo-1) mutations. J Clin Invest 1999;103:355-63.
597. Schneider P, Holler N, Bodmer JL, Hahne M, Frei K, Fontana A, Tschopp J. Conversion of membrane-bound Fas(CD95) ligand to its soluble form is associated with downregulation of its proapoptotic activity and loss of liver toxicity. J Exp Med 1998;187:1205-13.
598. Hohlbaum AM, Moe S, Marshak-Rothstein A. Opposing effects of transmembrane and soluble Fas ligand expression on inflammation and tumor cell survival. J Exp Med 2000;191:1209-20.
599. Tanaka M, Suda T, Yatomi T, Nakamura N, Nagata S. Lethal effect of recombinant human Fas ligand in mice pretreated with Propionibacterium acnes. J Immunol 1997;158:2303-9.
600. Ottonello L, Tortolina G, Amelotti M, Dallegri F. Soluble Fas ligand is chemotactic for human neutrophilic polymorphonuclear leukocytes. J Immunol 1999;162:3601-6.
601. Greenstein S, Barnard J, Zhou K, Fong M, Hendey B. Fas activation reduces neutrophil adhesion to endothelial cells. J Leukoc Biol 2000;68:715-22.
602. Hendey B, Zhu CL, Greenstein S. Fas activation opposes PMA-stimulated changes in the localization of PKCdelta: a mechanism for reducing neutrophil adhesion to endothelial cells. J Leukoc Biol 2002;71:863-70.

603. Shudo K, Kinoshita K, Imamura R, Fan H, Hasumoto K, Tanaka M, Nagata S, Suda T. The membrane-bound but not the soluble form of human Fas ligand is responsible for its inflammatory activity. Eur J Immunol 2001;31:2504-11.
604. Bossi G, Griffiths GM. Degranulation plays an essential part in regulating cell surface expression of Fas ligand in T cells and natural killer cells. Nat Med 1999;5:90-6.
605. Chen JJ, Sun Y, Nabel GJ. Regulation of the proinflammatory effects of Fas ligand (CD95L). Science 1998;282:1714-7.
606. Seino K, Iwabuchi K, Kayagaki N, Miyata R, Nagaoka I, Matsuzawa A, Fukao K, Yagita H, Okumura K. Chemotactic activity of soluble Fas ligand against phagocytes. J Immunol 1998;161:4484-8.
607. Peter ME, Krammer PH. Mechanisms of CD95 (APO-1/Fas)-mediated apoptosis. Curr Opin Immunol 1998;10:545-51.
608. Kim HJ, Park HJ, Park WS, Bae Y. CD43 cross-linking increases the Fas-induced apoptosis through induction of Fas aggregation in Jurkat T-cells. Exp Mol Med 2006;38:357-63.
609. Suda T. [Molecular mechanisms and biological roles of apoptosis and inflammation induced by Fas ligand]. Tanpakushitsu Kakusan Koso 1999;44:1477-86.
610. Seino K, Tun T, Ohshima N, Hamada H, Yoshino K, Ikeda S, Fukunaga K, Taniguchi H, Takada Y, Yuzawa K, Otsuka M, Todoroki T, Fukao K. Inhibition of CD95 ligand-mediated inflammation. Transplant Proc 2000;32:2038-9.
611. Jang S, Krammer PH, Salgame P. Lack of proapoptotic activity of soluble CD95 ligand is due to its failure to induce CD95 oligomers. J Interferon Cytokine Res 2003;23:441-7.
612. Sieg S, Smith D, Kaplan D. Differential activity of soluble versus cellular Fas ligand: regulation by an accessory molecule. Cell Immunol 1999;195:89-95.
613. Aoki K, Kurooka M, Chen JJ, Petryniak J, Nabel EG, Nabel GJ. Extracellular matrix interacts with soluble CD95L: retention and enhancement of cytotoxicity. Nat Immunol 2001;2:333-7.
614. Zanin-Zhorov A, Hershkoviz R, Hecht I, Cahalon L, Lider O. Fibronectin-associated Fas ligand rapidly induces opposing and time-dependent effects on the activation and apoptosis of T cells. J Immunol 2003;171:5882-9.
615. Holler N, Tardivel A, Kovacsovics-Bankowski M, Hertig S, Gaide O, Martinon F, Tinel A, Deperthes D, Calderara S, Schulthess T, Engel J, Schneider P, Tschopp J. Two adjacent trimeric Fas ligands are required for Fas signaling and formation of a death-inducing signaling complex. Mol Cell Biol 2003;23:1428-40.
616. Shiraishi T, Suzuyama K, Okamoto H, Mineta T, Tabuchi K, Nakayama K, Shimizu Y, Tohma J, Ogihara T, Naba H, Mochizuki H, Nagata S. Increased cytotoxicity of soluble Fas ligand by fusing isoleucine zipper motif. Biochem Biophys Res Commun 2004;322:197-202.
617. Proussakova OV, Rabaya NA, Moshnikova AB, Telegina ES, Turanov A, Nanazashvili MG, Beletsky IP. Oligomerization of soluble Fas antigen induces its cytotoxicity. J Biol Chem 2003;278:36236-41.
618. Schulte M, Reiss K, Lettau M, Maretzky T, Ludwig A, Hartmann D, de Strooper B, Janssen O, Saftig P. ADAM10 regulates FasL cell surface expression and

modulates FasL-induced cytotoxicity and activation-induced cell death. Cell Death Differ 2007;14:1040-9.
619. Berg D, Lehne M, Muller N, Siegmund D, Munkel S, Sebald W, Pfizenmaier K, Wajant H. Enforced covalent trimerization increases the activity of the TNF ligand family members TRAIL and CD95L. Cell Death Differ 2007;14:2021-34.
620. Suda T, Tanaka M, Miwa K, Nagata S. Apoptosis of mouse naive T cells induced by recombinant soluble Fas ligand and activation-induced resistance to Fas ligand. J Immunol 1996;157:3918-24.
621. Fingleton B, Vargo-Gogola T, Crawford HC, Matrisian LM. Matrilysin [MMP-7] expression selects for cells with reduced sensitivity to apoptosis. Neoplasia 2001;3:459-68.
622. Schneider P, Bodmer JL, Holler N, Mattmann C, Scuderi P, Terskikh A, Peitsch MC, Tschopp J. Characterization of Fas (Apo-1, CD95)-Fas ligand interaction. J Biol Chem 1997;272:18827-33.
623. Amara JF, Clackson T, Rivera VM, Guo T, Keenan T, Natesan S, Pollock R, Yang W, Courage NL, Holt DA, Gilman M. A versatile synthetic dimerizer for the regulation of protein-protein interactions. Proc Natl Acad Sci U S A 1997;94:10618-23.
624. Ali MH, Imperiali B. Protein oligomerization: how and why. Bioorg Med Chem 2005;13:5013-20.
625. Henkler F, Behrle E, Dennehy KM, Wicovsky A, Peters N, Warnke C, Pfizenmaier K, Wajant H. The extracellular domains of FasL and Fas are sufficient for the formation of supramolecular FasL-Fas clusters of high stability. J Cell Biol 2005;168:1087-98.
626. Siegel RM, Martin DA, Zheng L, Ng SY, Bertin J, Cohen J, Lenardo MJ. Death-effector filaments: novel cytoplasmic structures that recruit caspases and trigger apoptosis. J Cell Biol 1998;141:1243-53.
627. Hahne M, Peitsch MC, Irmler M, Schroter M, Lowin B, Rousseau M, Bron C, Renno T, French L, Tschopp J. Characterization of the non-functional Fas ligand of gld mice. Int Immunol 1995;7:1381-6.
628. Oyaizu N, Kayagaki N, Yagita H, Pahwa S, Ikawa Y. Requirement of cell-cell contact in the induction of Jurkat T cell apoptosis: the membrane-anchored but not soluble form of FasL can trigger anti-CD3-induced apoptosis in Jurkat T cells. Biochem Biophys Res Commun 1997;238:670-5.
629. Muraki M. Secretory expression of synthetic human Fas ligand extracellular domain gene in Pichia pastoris: influences of tag addition and N-glycosylation site deletion, and development of a purification method. Protein Expr Purif 2006;50:137-46.
630. Shechter Y. Selective oxidation and reduction of methionine residues in peptides and proteins by oxygen exchange between sulfoxide and sulfide. J Biol Chem 1986;261:66-70.
631. Schagger H, von Jagow G. Blue native electrophoresis for isolation of membrane protein complexes in enzymatically active form. Anal Biochem 1991;199:223-31.
632. Steinberg KP, Milberg JA, Martin TR, Maunder RJ, Cockrill BA, Hudson LD. Evolution of bronchoalveolar cell populations in the adult respiratory distress syndrome. Am J Respir Crit Care Med 1994;150:113-22.

633. Powell SR, Wang P, Divald A, Teichberg S, Haridas V, McCloskey TW, Davies KJ, Katzeff H. Aggregates of oxidized proteins (lipofuscin) induce apoptosis through proteasome inhibition and dysregulation of proapoptotic proteins. Free Radic Biol Med 2005;38:1093-101.
634. Stadtman ER, Moskovitz J, Levine RL. Oxidation of methionine residues of proteins: biological consequences. Antioxid Redox Signal 2003;5:577-82.
635. Vogt W. Oxidation of methionyl residues in proteins: tools, targets, and reversal. Free Radic Biol Med 1995;18:93-105.
636. Chao CC, Ma YS, Stadtman ER. Modification of protein surface hydrophobicity and methionine oxidation by oxidative systems. Proc Natl Acad Sci U S A 1997;94:2969-74.
637. Naskalski JW. Oxidative modification of protein structures under the action of myeloperoxidase and the hydrogen peroxide and chloride system. Ann Biol Clin (Paris) 1994;52:451-6.
638. Fu X, Kassim SY, Parks WC, Heinecke JW. Hypochlorous acid generated by myeloperoxidase modifies adjacent tryptophan and glycine residues in the catalytic domain of matrix metalloproteinase-7 (matrilysin): an oxidative mechanism for restraining proteolytic activity during inflammation. J Biol Chem 2003;278:28403-9.
639. Chapman AL, Winterbourn CC, Brennan SO, Jordan TW, Kettle AJ. Characterization of non-covalent oligomers of proteins treated with hypochlorous acid. Biochem J 2003;375:33-40.
640. Olszowski S, Olszowska E, Kusior D, Piwowarczyk M, Stelmaszynska T. Hypochlorite action on plasma fibronectin promotes its extended conformation in complexes with antibodies. J Protein Chem 2003;22:449-56.
641. Naskalski JW, Marcinkiewicz J, Drozdz R. Myeloperoxidase-mediated protein oxidation: its possible biological functions. Clin Chem Lab Med 2002;40:463-8.
642. Requena JR, Dimitrova MN, Legname G, Teijeira S, Prusiner SB, Levine RL. Oxidation of methionine residues in the prion protein by hydrogen peroxide. Arch Biochem Biophys 2004;432:188-95.
643. Stadtman ER, Van Remmen H, Richardson A, Wehr NB, Levine RL. Methionine oxidation and aging. Biochim Biophys Acta 2005;1703:135-40.
644. Temple A, Yen TY, Gronert S. Identification of specific protein carbonylation sites in model oxidations of human serum albumin. J Am Soc Mass Spectrom 2006;17:1172-80.
645. Fu X, Kassim SY, Parks WC, Heinecke JW. Hypochlorous acid oxygenates the cysteine switch domain of pro-matrilysin (MMP-7). A mechanism for matrix metalloproteinase activation and atherosclerotic plaque rupture by myeloperoxidase. J Biol Chem 2001;276:41279-87.
646. Tanaka T, Toujima S, Otani T, Minami S, Yamoto M, Umesaki N. Expression and function of activin receptors in human endometrial adenocarcinoma cells. Int J Oncol 2003;23:657-63.
647. Kondo T, Suda T, Fukuyama H, Adachi M, Nagata S. Essential roles of the Fas ligand in the development of hepatitis. Nat Med 1997;3:409-13.
648. Marsik C, Halama T, Cardona F, Wlassits W, Mayr F, Pleiner J, Jilma B. Regulation of Fas (APO-1, CD95) and Fas ligand expression in leukocytes during systemic inflammation in humans. Shock 2003;20:493-6.

649. Ayala A, Chung CS, Lomas JL, Song GY, Doughty LA, Gregory SH, Cioffi WG, LeBlanc BW, Reichner J, Simms HH, Grutkoski PS. Shock-induced neutrophil mediated priming for acute lung injury in mice: divergent effects of TLR-4 and TLR-4/FasL deficiency. Am J Pathol 2002;161:2283-94.
650. Chabot F, Mitchell JA, Gutteridge JM, Evans TW. Reactive oxygen species in acute lung injury. Eur Respir J 1998;11:745-57.
651. Cross CE, Forte T, Stocker R, Louie S, Yamamoto Y, Ames BN, Frei B. Oxidative stress and abnormal cholesterol metabolism in patients with adult respiratory distress syndrome. J Lab Clin Med 1990;115:396-404.
652. Fujishima S, Morisaki H, Ishizaka A, Kotake Y, Miyaki M, Yoh K, Sekine K, Sasaki J, Tasaka S, Hasegawa N, Kawai Y, Takeda J, Aikawa N. Neutrophil elastase and systemic inflammatory response syndrome in the initiation and development of acute lung injury among critically ill patients. Biomed Pharmacother 2008;62:333-8.
653. Idell S, Kucich U, Fein A, Kueppers F, James HL, Walsh PN, Weinbaum G, Colman RW, Cohen AB. Neutrophil elastase-releasing factors in bronchoalveolar lavage from patients with adult respiratory distress syndrome. Am Rev Respir Dis 1985;132:1098-105.
654. Kaynar AM, Houghton AM, Lum EH, Pitt BR, Shapiro SD. Neutrophil elastase is needed for neutrophil emigration into lungs in ventilator-induced lung injury. Am J Respir Cell Mol Biol 2008;39:53-60.
655. Itoh N, Tsujimoto Y, Nagata S. Effect of bcl-2 on Fas antigen-mediated cell death. J Immunol 1993;151:621-7.
656. Kawahara A, Kobayashi T, Nagata S. Inhibition of Fas-induced apoptosis by Bcl-2. Oncogene 1998;17:2549-54.
657. Baumler C, Duan F, Onel K, Rapaport B, Jhanwar S, Offit K, Elkon KB. Differential recruitment of caspase 8 to cFlip confers sensitivity or resistance to Fas-mediated apoptosis in a subset of familial lymphoma patients. Leuk Res 2003;27:841-51.
658. Hyer ML, Sudarshan S, Kim Y, Reed JC, Dong JY, Schwartz DA, Norris JS. Downregulation of c-FLIP sensitizes DU145 prostate cancer cells to Fas-mediated apoptosis. Cancer Biol Ther 2002;1:401-6.
659. Irmler M, Thome M, Hahne M, Schneider P, Hofmann K, Steiner V, Bodmer JL, Schroter M, Burns K, Mattmann C, Rimoldi D, French LE, Tschopp J. Inhibition of death receptor signals by cellular FLIP. Nature 1997;388:190-5.
660. Jin TG, Kurakin A, Benhaga N, Abe K, Mohseni M, Sandra F, Song K, Kay BK, Khosravi-Far R. Fas-associated protein with death domain (FADD)-independent recruitment of c-FLIPL to death receptor 5. J Biol Chem 2004;279:55594-601.
661. Scaffidi C, Schmitz I, Krammer PH, Peter ME. The role of c-FLIP in modulation of CD95-induced apoptosis. J Biol Chem 1999;274:1541-8.
662. Leu JI, Crissey MA, Taub R. Massive hepatic apoptosis associated with TGF-beta1 activation after Fas ligand treatment of IGF binding protein-1-deficient mice. J Clin Invest 2003;111:129-39.
663. Tanaka Y, Sohda T, Matsuo K, Anan A, Irie M, Takeyama Y, Iwata K, Shakado S, Sakisaka S. Vascular endothelial growth factor reduces Fas-mediated acute liver injury in mice. J Gastroenterol Hepatol 2008;23:e207-11.
664. Schneider-Brachert W, Tchikov V, Neumeyer J, Jakob M, Winoto-Morbach S, Held-Feindt J, Heinrich M, Merkel O, Ehrenschwender M, Adam D, Mentlein

R, Kabelitz D, Schutze S. Compartmentalization of TNF receptor 1 signaling: internalized TNF receptosomes as death signaling vesicles. Immunity 2004;21:415-28.
665. Scott FL, Stec B, Pop C, Dobaczewska MK, Lee JJ, Monosov E, Robinson H, Salvesen GS, Schwarzenbacher R, Riedl SJ. The Fas-FADD death domain complex structure unravels signalling by receptor clustering. Nature 2009;457:1019-22.
666. Parks WC, Lopez-Boado YS, Wilson CL. Matrilysin in epithelial repair and defense. Chest 2001;120:36S-41S.
667. Busiek DF, Baragi V, Nehring LC, Parks WC, Welgus HG. Matrilysin expression by human mononuclear phagocytes and its regulation by cytokines and hormones. J Immunol 1995;154:6484-91.
668. Busiek DF, Ross FP, McDonnell S, Murphy G, Matrisian LM, Welgus HG. The matrix metalloprotease matrilysin (PUMP) is expressed in developing human mononuclear phagocytes. J Biol Chem 1992;267:9087-92.
669. Workalemahu G, Foerster M, Kroegel C. Expression of metalloproteinase-7 (matrilysin) in human blood and bronchoalveolar gamma/delta T-lymphocytes. Selective upregulation by the soluble non-peptidic mycobacterial phosphoantigen (isopentenyl pyrophosphate). J Cell Physiol 2006;207:67-74.
670. Swee M, Wilson CL, Wang Y, McGuire JK, Parks WC. Matrix metalloproteinase-7 (matrilysin) controls neutrophil egress by generating chemokine gradients. J Leukoc Biol 2008;83:1404-12.
671. Tanaka M, Suda T, Haze K, Nakamura N, Sato K, Kimura F, Motoyoshi K, Mizuki M, Tagawa S, Ohga S, Hatake K, Drummond AH, Nagata S. Fas ligand in human serum. Nat Med 1996;2:317-22.
672. Eramo A, Sargiacomo M, Ricci-Vitiani L, Todaro M, Stassi G, Messina CG, Parolini I, Lotti F, Sette G, Peschle C, De Maria R. CD95 death-inducing signaling complex formation and internalization occur in lipid rafts of type I and type II cells. Eur J Immunol 2004;34:1930-40.
673. Schutze S, Schneider-Brachert W. Impact of TNF-R1 and CD95 Internalization on Apoptotic and Antiapoptotic Signaling. Results Probl Cell Differ 2009.
674. Schutze S, Tchikov V, Schneider-Brachert W. Regulation of TNFR1 and CD95 signalling by receptor compartmentalization. Nat Rev Mol Cell Biol 2008;9:655-62.
675. Lamb NJ, Gutteridge JM, Baker C, Evans TW, Quinlan GJ. Oxidative damage to proteins of bronchoalveolar lavage fluid in patients with acute respiratory distress syndrome: evidence for neutrophil-mediated hydroxylation, nitration, and chlorination. Crit Care Med 1999;27:1738-44.
676. Houghton J, Macera-Bloch LS, Harrison L, Kim KH, Korah RM. Tumor necrosis factor alpha and interleukin 1beta up-regulate gastric mucosal Fas antigen expression in Helicobacter pylori infection. Infect Immun 2000;68:1189-95.
677. Ricciardolo FL, Di Stefano A, Sabatini F, Folkerts G. Reactive nitrogen species in the respiratory tract. Eur J Pharmacol 2006;533:240-52.
678. Zhu S, Ware LB, Geiser T, Matthay MA, Matalon S. Increased levels of nitrate and surfactant protein a nitration in the pulmonary edema fluid of patients with acute lung injury. Am J Respir Crit Care Med 2001;163:166-72.

679. Asimakopoulos G, Smith PL, Ratnatunga CP, Taylor KM. Lung injury and acute respiratory distress syndrome after cardiopulmonary bypass. Ann Thorac Surg 1999;68:1107-15.
680. Elkington PT, Friedland JS. Matrix metalloproteinases in destructive pulmonary pathology. Thorax 2006;61:259-66.
681. Moraes TJ, Chow CW, Downey GP. Proteases and lung injury. Crit Care Med 2003;31:S189-94.
682. Abul H, Abul A, Khan I, Mathew TC, Ayed A, Al-Athary E. Levels of IL-8 and myeloperoxidase in the lungs of pneumonia patients. Mol Cell Biochem 2001;217:107-12.
683. Mathy-Hartert M, Damas P, Nys M, Deby-Dupont G, Canivet JL, Ledoux D, Lamy M. Nitrated proteins in bronchoalveolar lavage fluid of patients at risk of ventilator-associated bronchopneumonia. Eur Respir J 2000;16:296-301.
684. Nys M, Deby-Dupont G, Habraken Y, Legrand-Poels S, Kohnen S, Ledoux D, Canivet JL, Damas P, Lamy M. Bronchoalveolar lavage fluids of ventilated patients with acute lung injury activate NF-kappaB in alveolar epithelial cell line: role of reactive oxygen/nitrogen species and cytokines. Nitric Oxide 2003;9:33-43.
685. Shacter E, Lopez RL, Pati S. Inhibition of the myeloperoxidase-H2O2-Cl- system of neutrophils by indomethacin and other non-steroidal anti-inflammatory drugs. Biochem Pharmacol 1991;41:975-84.
686. Crimi E, Sica V, Slutsky AS, Zhang H, Williams-Ignarro S, Ignarro LJ, Napoli C. Role of oxidative stress in experimental sepsis and multisystem organ dysfunction. Free Radic Res 2006;40:665-72.
687. Heffner JE, Matthay MA. The case for oxidants in the adult respiratory distress syndrome--"elementary, Dr. Watson". J Lab Clin Med 1990;115:394-5.
688. Lykens MG, Davis WB, Pacht ER. Antioxidant activity of bronchoalveolar lavage fluid in the adult respiratory distress syndrome. Am J Physiol 1992;262:L169-75.
689. Rahman I, MacNee W. Oxidative stress and regulation of glutathione in lung inflammation. Eur Respir J 2000;16:534-54.
690. Ruffmann R. Reactive oxygen species in acute lung injury. Eur Respir J 1998;12:1486.
691. Syrkina O, Jafari B, Hales CA, Quinn DA. Oxidant stress mediates inflammation and apoptosis in ventilator-induced lung injury. Respirology 2008;13:333-40.
692. Wu M. DNA repair proteins as molecular therapeutics for oxidative and alkylating lung injury. Curr Gene Ther 2005;5:225-36.
693. Park SJ, Ciccone SL, Beck BD, Hwang B, Freie B, Clapp DW, Lee SH. Oxidative stress/damage induces multimerization and interaction of Fanconi anemia proteins. J Biol Chem 2004;279:30053-9.
694. Atwood CS, Huang X, Khatri A, Scarpa RC, Kim YS, Moir RD, Tanzi RE, Roher AE, Bush AI. Copper catalyzed oxidation of Alzheimer Abeta. Cell Mol Biol (Noisy-le-grand) 2000;46:777-83.
695. Choi J, Sullards MC, Olzmann JA, Rees HD, Weintraub ST, Bostwick DE, Gearing M, Levey AI, Chin LS, Li L. Oxidative damage of DJ-1 is linked to sporadic Parkinson and Alzheimer diseases. J Biol Chem 2006;281:10816-24.

696. Mirzaei H, Regnier F. Protein:protein aggregation induced by protein oxidation. J Chromatogr B Analyt Technol Biomed Life Sci 2008;873:8-14.
697. Dalle-Donne I, Rossi R, Giustarini D, Gagliano N, Lusini L, Milzani A, Di Simplicio P, Colombo R. Actin carbonylation: from a simple marker of protein oxidation to relevant signs of severe functional impairment. Free Radic Biol Med 2001;31:1075-83.
698. Hung A, Griffin MD, Howlett GJ, Yarovsky I. Effects of oxidation, pH and lipids on amyloidogenic peptide structure: implications for fibril formation? Eur Biophys J 2008;38:99-110.
699. Squier TC. Oxidative stress and protein aggregation during biological aging. Exp Gerontol 2001;36:1539-50.
700. Grune T, Reinheckel T, Davies KJ. Degradation of oxidized proteins in mammalian cells. FASEB J 1997;11:526-34.
701. Davies KJ, Delsignore ME, Lin SW. Protein damage and degradation by oxygen radicals. II. Modification of amino acids. J Biol Chem 1987;262:9902-7.
702. Davies G, Jiang WG, Mason MD. Matrilysin mediates extracellular cleavage of E-cadherin from prostate cancer cells: a key mechanism in hepatocyte growth factor/scatter factor-induced cell-cell dissociation and in vitro invasion. Clin Cancer Res 2001;7:3289-97.
703. Berlett BS, Stadtman ER. Protein oxidation in aging, disease, and oxidative stress. J Biol Chem 1997;272:20313-6.
704. Bargnoux AS, Morena M, Badiou S, Dupuy AM, Canaud B, Cristol JP, Groupe de travail de la SBdfredlir. [Carbonyl stress and oxidatively modified proteins in chronic renal failure]. Ann Biol Clin (Paris) 2009;67:153-8.
705. Blackburn AC, Doe WF, Buffinton GD. Protein carbonyl formation on mucosal proteins in vitro and in dextran sulfate-induced colitis. Free Radic Biol Med 1999;27:262-70.
706. Levine RL, Moskovitz J, Stadtman ER. Oxidation of methionine in proteins: roles in antioxidant defense and cellular regulation. IUBMB Life 2000;50:301-7.
707. Levine RL, Berlett BS, Moskovitz J, Mosoni L, Stadtman ER. Methionine residues may protect proteins from critical oxidative damage. Mech Ageing Dev 1999;107:323-32.
708. Coleman LG, Jr., Polanowska-Grabowska RK, Marcinkiewicz M, Gear AR. LDL oxidized by hypochlorous acid causes irreversible platelet aggregation when combined with low levels of ADP, thrombin, epinephrine, or macrophage-derived chemokine (CCL22). Blood 2004;104:380-9.
709. Jerlich A, Fabjan JS, Tschabuschnig S, Smirnova AV, Horakova L, Hayn M, Auer H, Guttenberger H, Leis HJ, Tatzber F, Waeg G, Schaur RJ. Human low density lipoprotein as a target of hypochlorite generated by myeloperoxidase. Free Radic Biol Med 1998;24:1139-48.
710. Olszowski S, Olszowska E, Stelmaszynska T, Krawczyk A, Marcinkiewicz J, Baczek N. Oxidative modification of ovalbumin. Acta Biochim Pol 1996;43:661-72.
711. Kanski J, Aksenova M, Butterfield DA. The hydrophobic environment of Met35 of Alzheimer's Abeta(1-42) is important for the neurotoxic and oxidative properties of the peptide. Neurotox Res 2002;4:219-23.
712. Murakami K, Irie K, Ohigashi H, Hara H, Nagao M, Shimizu T, Shirasawa T. Formation and stabilization model of the 42-mer Abeta radical: implications for

the long-lasting oxidative stress in Alzheimer's disease. J Am Chem Soc 2005;127:15168-74.
713. Narayanan S, Kamps B, Boelens WC, Reif B. alphaB-crystallin competes with Alzheimer's disease beta-amyloid peptide for peptide-peptide interactions and induces oxidation of Abeta-Met35. FEBS Lett 2006;580:5941-6.
714. Peskin AV, Winterbourn CC. Taurine chloramine is more selective than hypochlorous acid at targeting critical cysteines and inactivating creatine kinase and glyceraldehyde-3-phosphate dehydrogenase. Free Radic Biol Med 2006;40:45-53.
715. Dalle-Donne I, Rossi R, Giustarini D, Gagliano N, Di Simplicio P, Colombo R, Milzani A. Methionine oxidation as a major cause of the functional impairment of oxidized actin. Free Radic Biol Med 2002;32:927-37.
716. Szuchman-Sapir AJ, Pattison DI, Ellis NA, Hawkins CL, Davies MJ, Witting PK. Hypochlorous acid oxidizes methionine and tryptophan residues in myoglobin. Free Radic Biol Med 2008;45:789-98.
717. Thomas EL, Jefferson MM, Grisham MB. Myeloperoxidase-catalyzed incorporation of amines into proteins: role of hypochlorous acid and dichloramines. Biochemistry 1982;21:6299-308.
718. Stadtman ER, Moskovitz J, Berlett BS, Levine RL. Cyclic oxidation and reduction of protein methionine residues is an important antioxidant mechanism. Mol Cell Biochem 2002;234-235:3-9.
719. Stadtman ER. Protein oxidation in aging and age-related diseases. Ann N Y Acad Sci 2001;928:22-38.
720. Stamler JS, Hausladen A. Oxidative modifications in nitrosative stress. Nat Struct Biol 1998;5:247-9.
721. Schachter H, Dixon GH. PREFERENTIAL OXIDATION OF THE METHIONINE RESIDUE NEAR THE ACTIVE SITE OF CHYMOTRYPSIN. J Biol Chem 1964;239:813-29.
722. Schoneich C. Methionine oxidation by reactive oxygen species: reaction mechanisms and relevance to Alzheimer's disease. Biochim Biophys Acta 2005;1703:111-9.
723. DePaz RA, Barnett CC, Dale DA, Carpenter JF, Gaertner AL, Randolph TW. The excluding effects of sucrose on a protein chemical degradation pathway: methionine oxidation in subtilisin. Arch Biochem Biophys 2000;384:123-32.
724. Hoshi T, Heinemann S. Regulation of cell function by methionine oxidation and reduction. J Physiol 2001;531:1-11.
725. Shao B, Cavigiolio G, Brot N, Oda MN, Heinecke JW. Methionine oxidation impairs reverse cholesterol transport by apolipoprotein A-I. Proc Natl Acad Sci U S A 2008;105:12224-9.
726. Fliss H. Oxidation of proteins in rat heart and lungs by polymorphonuclear leukocyte oxidants. Mol Cell Biochem 1988;84:177-88.
727. Fliss H, Menard M. Rapid neutrophil accumulation and protein oxidation in irradiated rat lungs. J Appl Physiol 1994;77:2727-33.
728. Maier KL, Leuschel L, Costabel U. Increased oxidized methionine residues in BAL fluid proteins in acute or chronic bronchitis. Eur Respir J 1992;5:651-8.
729. Fliss H, Weissbach H, Brot N. Oxidation of methionine residues in proteins of activated human neutrophils. Proc Natl Acad Sci U S A 1983;80:7160-4.

730. Kim HY, Gladyshev VN. Methionine sulfoxide reduction in mammals: characterization of methionine-R-sulfoxide reductases. Mol Biol Cell 2004;15:1055-64.
731. Hansel A, Kuschel L, Hehl S, Lemke C, Agricola HJ, Hoshi T, Heinemann SH. Mitochondrial targeting of the human peptide methionine sulfoxide reductase (MSRA), an enzyme involved in the repair of oxidized proteins. FASEB J 2002;16:911-3.
732. Hansel A, Jung S, Hoshi T, Heinemann SH. A second human methionine sulfoxide reductase (hMSRB2) reducing methionine-R-sulfoxide displays a tissue expression pattern distinct from hMSRB1. Redox Rep 2003;8:384-8.
733. Stadtman ER. Cyclic oxidation and reduction of methionine residues of proteins in antioxidant defense and cellular regulation. Arch Biochem Biophys 2004;423:2-5.
734. Hansel A, Heinemann SH, Hoshi T. Heterogeneity and function of mammalian MSRs: enzymes for repair, protection and regulation. Biochim Biophys Acta 2005;1703:239-47.